



PHD

Vaccination against cotinine as a potential approach to smoking cessation

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Award date:
2007

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Vaccination against cotinine as a potential approach to smoking cessation

Jennifer Lynne Oliver

**A thesis submitted for the degree of
Doctor of Philosophy**

**University of Bath
Department of Biology & Biochemistry
December 2007**

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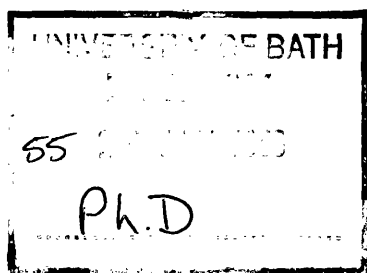
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Acknowledgments

There are many people, who have supported me in different ways over the last four years, and it is impossible to pay tribute to everyone individually; however, I would like to acknowledge some of the key contributors (in no particular order):

Firstly, I am grateful to GlaxoSmithKline and the Medical Research Council for funding this PhD. Also, Rick Chan deserves a big thank you for his ability to find extra funds in times of need.

"Merci beaucoup" to Rémi Palmantier for his continued support and enthusiasm, and to all those at GSK Biologicals in Belgium for making me feel welcome and enlightening me on the production process for the vaccine conjugates. [To whoever mislabelled that last batch of conjugates – I've forgiven you...just about!] "Grazie mille" also to Christian Heidbreder and his team at GSK Verona for their advice on the locomotor experiments.

Paul Mitchell and Graham were a big help when getting to grips with the locomotor equipment at Bath Uni, and Stephen Husbands kindly synthesised the new batch of cotinine derivative. And of course a huge thank you must go to all the NACWOs in Bath and Newcastle – I couldn't have done it without you!

I could not have wished for better supervisors; Sue – thanks ever so much for your patience, support and encouragement, and for believing in me. I am also exceedingly grateful to Paul Barnett for his "can do" approach to the project, and for enabling me to collaborate with so many people at GSK & beyond.

This PhD would not have been the same without the time I spent in Newcastle; many thanks to Mohammed for giving me the opportunity to try every behavioural paradigm known to man and for helping me through a seemingly endless IVSA experiment. (By the way, you still need to make good your promise to go salsa dancing with me at some point!)

"Cheers" to all the members of the Wonnacott lab, past and present, as well as to the Newcastle gang (especially Lynsay), for the mutual support and constant supply of tea & biscuits/chocolate.

"Muchas gracias" to the many salseros & salseras that have become firm friends over the years – without the opportunity to let off steam on the dance floor, I doubt I could have stayed sane (well, almost!). Thanks also to the committee(s) and members of my beloved SalsaSoc – please take good care of my baby!

One person who has been there for me ever since Freshers' Week eight years ago is Phil; thanks for teaching me to dance – it has changed my life – and for being my best mate and lunch buddy for so many years.

And last – but by no means least – I would not be where I am today without all the love and support provided in so many different ways by my parents and sisters over the past twenty-odd years.

Thank you all very much!

Summary

Cigarette smoking is the single largest cause of preventable death and disease. Each year ~50% of smokers attempt to quit, but less than 5% are successful, despite using pharmacotherapy. Immunotherapy is being explored as a new approach to smoking cessation; several anti-nicotine vaccines are being developed, which reduce nicotine reinforcement. Anti-nicotine antibodies bind nicotine, preventing it from entering the brain and acting on nicotinic acetylcholine receptors (nAChRs). However, this approach also renders nicotine replacement therapy (NRT) ineffective.

Cotinine, the major metabolite of nicotine, is a weak agonist at nAChRs, inducing striatal dopamine release; there is also evidence that it antagonises nicotine's actions. An anti-cotinine vaccine would reduce such antagonism by preventing cotinine from passing the blood-brain-barrier, and sequestering it in the bloodstream. This should enhance the efficacy of NRT and assist with a reduce-to-quit approach to smoking cessation.

Here we characterise the immune response to anti-cotinine vaccination and study the impact of vaccination on measures of nicotine dependence.

Anti-cotinine vaccination was found to be safe and immunogenic, with regular booster injections required to maintain antibody levels. Antibodies raised were specific for cotinine, retaining it in the blood. Upregulation of nAChRs by chronic nicotine administration was not increased after vaccination, and no effects on conditioned taste aversion were observed. However, increases in locomotor activity induced by repeated nicotine administration occurred earlier and were more pronounced in vaccinated rats. And in a model of nicotine withdrawal, abstinence scores were increased in vaccinated rats, due to the removal of cotinine-mediated antagonism of nicotine's actions.

Antibody levels measured here were not as high as titres in studies of anti-nicotine vaccines; however, in view of the effects observed with comparatively low titres, vaccination against cotinine could offer a new approach to smoking cessation, provided higher titres of cotinine-specific antibodies can be achieved.

Publications and Communications

Publications

- Oliver JL, Pashmi G, Barnett P, Mettens P, Biemans R, Monteyne P, Palmantier R, Gallagher T, Ramaya S, Wonnacott S. (2007) Development of an anti-cotinine vaccine to potentiate nicotine-based smoking cessation strategies. *Vaccine*; **25**: 7354-7362

Communications

- Oliver JL, Barnett P, Heidbreder C, Palmantier R, Wonnacott S. (2005) Effect of anti-cotinine immunotherapy in nicotine behavioural studies. Society for Neuroscience 35th Ann Meeting, Poster 113.9

- Oliver JL, Barnett P, Heidbreder C, Palmantier R, Wonnacott S. (2005) Targetting cotinine to aid smoking cessation. SRNT Joint Annual & European Meeting, Prague (CZ), Poster 1-55

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List of abbreviations

| | |
|-------------------------|---|
| [¹²⁵ I]αBgt | [¹²⁵ I]α-Bungarotoxin |
| [³ H]MLA | [³ H]methyllaconitine |
| 3-HC | (3'R,5'S)- <i>trans</i> -3'-hydroxycotinine |
| 5-HT | serotonin |
| 6-OHDA | 6-hydroxydopamine |
| ACh | acetylcholine |
| AChBP | acetylcholine binding protein |
| Alum | aluminium hydroxide |
| ANOVA | analysis of variance |
| AUC | area under the curve |
| Aβ | amyloid β |
| BSA | bovine serum albumin |
| CNS | central nervous system |
| CotSH | <i>trans</i> -4-thio-cotinine |
| CTA | conditioned taste aversion |
| CYP | cytochrome P450 enzyme |
| DA | dopamine |
| DHβE | dihydro-β-erythroidine |
| ER | endoplasmatic reticulum |
| F344 | Fisher rat strain |
| FDA | Food & Drug Administration (US) |
| FR | fixed ratio (self-administration responding schedule) |
| GABA | γ-aminobutyric acid |
| i.v. | intravenous route of administration |
| IgG | immunoglobulin (isotype G) |
| ISCOMS | immunomodulatory complexes |
| IVSA | intravenous self-administration |
| LDT | laterodorsal tegmental nucleus |
| LE | Long-Evans rat strain |
| mAb | monoclonal antibody |
| MAO-B | monoamine oxidase B |
| MBP | mannose-binding protein |

| | |
|---------------------|--|
| MHC | major histocompatibility complex |
| NA | noradrenalin |
| NAcc | nucleus accumbens |
| nAChR | nicotinic acetylcholine receptor |
| NDMA | N-nitrosodimethylamine |
| NK cells | natural killer cells |
| NMDA | N-methyl-D-aspartate |
| NNK | 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone |
| NRT | nicotine replacement therapy |
| OMP | osmotic minipump |
| PBS | phosphate-buffered saline |
| PCP | phencyclidine |
| PFC | prefrontal cortex |
| s.c. | subcutaneous route of administration |
| S.E.M. | standard error of the mean |
| SD | Sprague-Dawley rat strain |
| ST | smokeless tobacco |
| TA-CD | anti-cocaine vaccine (Xenova) |
| TPP | tegmental pedunculopontine nucleus |
| TSNAs | tobacco specific nitrosamines |
| TT | tetanus toxoid |
| TT-CotSH | <i>trans</i> -4-thio-cotinine coupled to tetanus toxoid (active conjugate) |
| TT-Cysteine | cysteine coupled to tetanus toxoid (control conjugate) |
| VOCC | voltage-operated calcium channel |
| VTA | ventral tegmental area |
| $\alpha 4\beta 2^*$ | $\alpha 4\beta 2$ -subunit containing receptor |

Chapter 1

Introduction

Chapter 1

Introduction

1.1 Nicotine

Nicotine is a naturally occurring alkaloid found in plants, such as tobacco (*Nicotiana tabacum*). It is a tertiary amine consisting of a pyridine and a pyrrolidine ring (Benowitz 1996, Schneider et al 2001, Yildiz 2004, Tutka et al 2005) and was first isolated as the major alkaloid in tobacco in 1828. (S)-nicotine is the main stereoisomer present in tobacco, whereas (R)-nicotine is found only in small quantities in cigarette smoke. Nicotine is the primary psychoactive component of tobacco, and addiction to nicotine is generally considered to be the reason why smokers find it so hard to quit (see section 1.6). Nicotine can have both stimulant and depressant effects, affecting mood, performance, cognitive function, as well as producing pleasure, reducing hunger and relieving anxiety and depression (Benowitz 1996, Schneider et al 2001, Yildiz 2004, Tutka et al 2005). However, whether this is a result of the positive actions of nicotine, or due to the relief of withdrawal symptoms, or both is unclear (see sections 1.4.2, 1.6 & 4.5).

In humans, nicotine is obtained through smoked (mainly cigarettes) or smoke-free (snuff, chewing tobacco, snus) tobacco products (see section 1.5), or from nicotine replacement therapy (NRT) products (see section 1.6.1.1). Nicotine can be absorbed through the oral cavity, skin, lung, urinary bladder and gastrointestinal tract. Absorption is pH-dependent: smoke from cigarettes has an acidic pH (~5.5), which means that nicotine is mostly ionised and does not readily permeate cell membranes (Yildiz 2004, Tutka et al 2005). However, in the lungs nicotine is buffered to a physiological pH and is rapidly absorbed, so that approximately a third of the nicotine inhaled passes into the bloodstream. In smokeless tobacco (snuff, chewing tobacco, etc) users and smokers who do not inhale, absorption occurs mainly through the oral mucosa. At an alkaline pH, such as in smoke from tobacco in pipes, nicotine is mostly non-ionised and easily crosses the oral mucosa (Yildiz 2004, Tutka et al 2005).

Nicotine has a half-life of approximately 1-2 hours and, once absorbed, is extensively metabolised by the liver to a number of major and minor metabolites, as discussed in section 1.2 of this thesis (Benowitz 1996, Schneider et al 2001, Yildiz 2004, Tutka et al 2005).

1.1.1 Nicotine administration in animal models of nicotine dependence

One of the main issues associated with preclinical models of nicotine addiction, is the question of how to give animals nicotine in a meaningful way. Smokers achieve a high dose of nicotine very quickly after each cigarette (see section 1.5.1), with levels declining gradually between cigarettes. Despite these repeated peaks and troughs, smokers maintain a relatively constant plasma nicotine level throughout their waking hours, leading to prolonged desensitisation of nAChRs (see section 1.3.2). During overnight abstinence plasma levels decrease, allowing the receptors to re-sensitise.

- Intravenous administration via implanted cannulae most closely mimics the pharmacokinetics of nicotine observed in smokers: repeated sudden peaks, followed by a gradual reduction in plasma nicotine levels in between cigarettes. This route of administration is used in self-administration experiments (see Chapter 4), however it is not suitable for a lot of other experimental setups, as it is quite invasive because of the surgery required to implant the intravenous cannula.

- Subcutaneous or intraperitoneal injections are probably one of the easiest and least stressful ways of administering nicotine to animals. Injections are usually given once or twice a day, which does not very accurately reflect the repeated administration seen in patients smoking cigarettes throughout the day. Usually the doses of nicotine given with each injection are also relatively large compared to intravenous administration, in order to be able to achieve the desired longer-term minimum plasma levels.

- Chronic administration of nicotine via subcutaneous osmotic minipumps is not a very accurate reflection of smoking behaviour, as nicotine is delivered at a steady rate and continuously for several days/weeks, rather than in frequent bursts. However, it does reproduce the steady state nicotine levels achieved by smokers (Sanderson et al 1993, Rowell & Li 1997), albeit without the overnight

abstinence, and mimics the situation of a patient using transdermal nicotine patches during a quit attempt quite well. There are differences in the route of administration (subcutaneous versus transdermal), though, as well as in the dosing pattern, with nicotine being delivered continuously with no overnight abstinence, as would be the case for a patient using NRT.

- Transdermal nicotine patches are not very practical for use on animals, as they can be removed/chewed by the individual or a cage mate, and fur usually has to be shaved prior to application in order to achieve a reasonable adhesion of the patch.

- A further alternative is the administration of nicotine in drinking water, which is often flavoured to disguise the aversive taste of nicotine. It has the advantage of being self-administered by the animal and, as surgical implantation of osmotic pumps is not very convenient due to their size, it is frequently used with mice (Brunzell et al 2006). The disadvantage here is that the experimenter has no direct control over the amount consumed or the dosing pattern, and at higher concentrations animals appear to dislike the taste of nicotine-containing solutions.

1.2 Nicotine metabolism

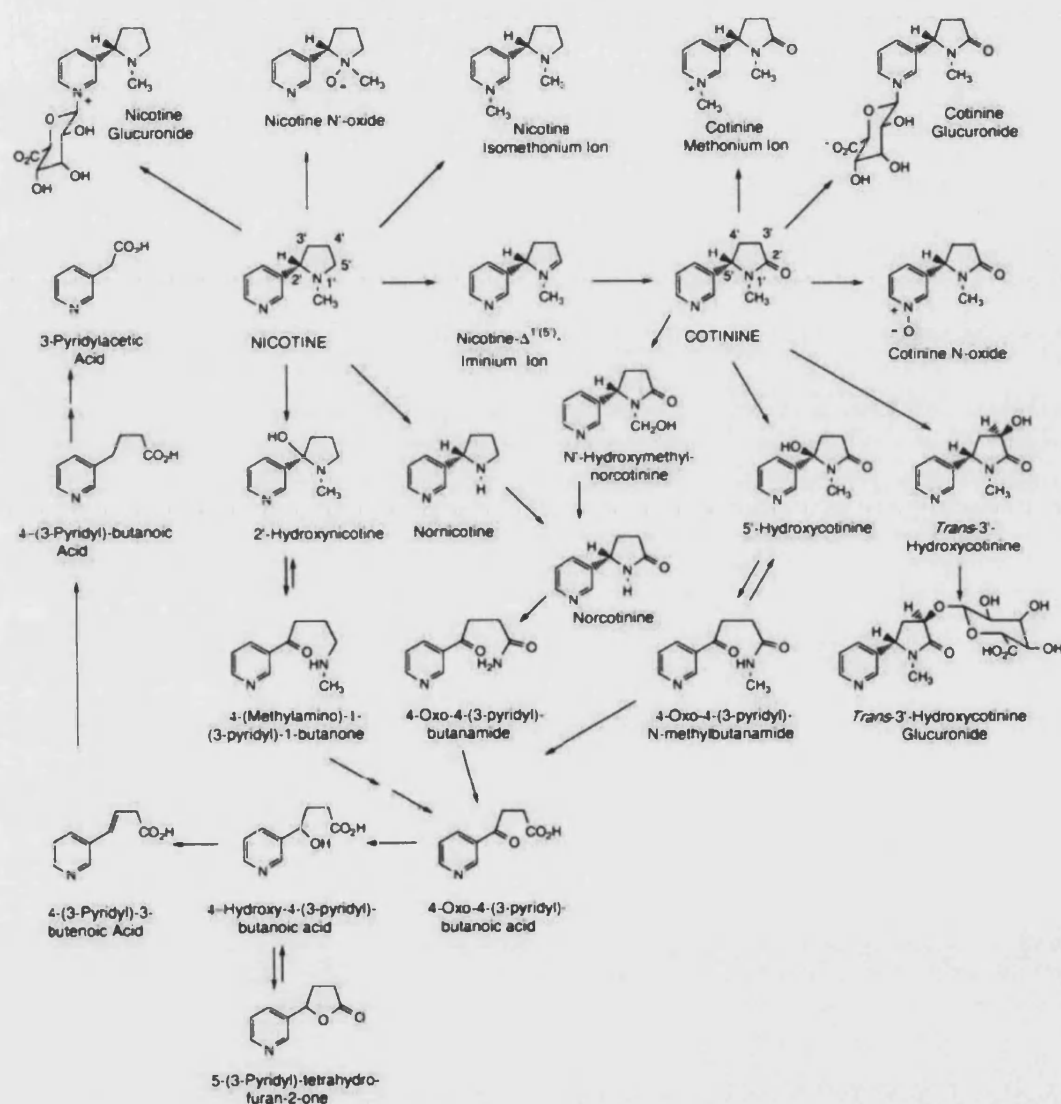
1.2.1 Enzymes & metabolic pathways involved in nicotine metabolism

The pathways involved in the metabolism of nicotine (and cotinine) are summarised in Figure 1.1. In humans, approximately 70-80% of nicotine is metabolised to cotinine by C-oxidation involving members of the cytochrome P-450 (CYP) enzyme family (Siu & Tyndale 2007, Yildiz 2004, Benowitz 1996). CYPs are mixed function oxidases, which are predominantly expressed in the liver and biotransform drugs, endogenous compounds, dietary constituents, and environmental toxins (Howard et al 2001).

High levels of CYPs have been observed in the liver, but they have also been located in other tissues, including lung, kidney and brain, indicating that some local metabolism of nicotine might also occur (Howard et al 2003). Exposure to low doses of nicotine for 7 days or more leads to an induction of CYPs in rat liver, lung, kidney and brain, which persists for up to 18 hrs in the liver

(Anandattheerthavarada et al 1993, Iba et al 1999, Miksys et al 2000, Howard et al 2001 & 2003). CYP levels also appear to be elevated in the brains and lungs of smokers compared to non-smokers (Iba et al 1999, Howard et al 2003). Chronic administration of cotinine, on the other hand, does not appear to increase CYP levels in rats (Micu et al 2003).

Figure 1.1: Pathways of nicotine and cotinine metabolism



Summary of nicotine and cotinine metabolism in man (from Hukkanen et al 2005)

In humans the CYP2A6 isoform, found primarily in the liver, mediates approximately 90% of the conversion of nicotine to cotinine (Yildiz 2004, Siu et al 2007). This conversion is a two-step process, involving the initial formation of

nicotine- Δ -1'(5')iminium ions, which are then converted into cotinine by the cytosolic enzyme aldehyde oxidase. Cotinine is then extensively metabolised, again by CYP2A6, to trans-3'-hydroxycotinine, which is the most abundant metabolite found in urine (Benowitz 1996, Siu & Tyndale 2007). The remaining nicotine is either transformed to other minor metabolites, such as nicotine-N'-oxide (~4%), or excreted unchanged. Nicotine and cotinine also undergo N-glucuronidation, while 3'-hydroxycotinine is subject to O-glucuronidation (Benowitz 1996).

Genetic polymorphism of CYP isoforms is observed in humans, and it has been suggested that the efficiency of the conversion of nicotine to cotinine and resulting variations in nicotine metabolism are at least partly due to such polymorphisms (Yildiz 2004, Tutka et al 2005). Metabolism of cotinine appears to be slower in black than in white smokers, for example, because of both slower oxidative metabolism of nicotine to cotinine via CYP2A6, and slower N-glucuronidation (Perez-Stable et al 1998, Pianezza et al 1998, Benowitz et al 1999). As this is the primary route for nicotine metabolism, alterations in the amount or function of CYP2A6 significantly affect plasma nicotine levels during smoking and nicotine replacement therapy (NRT), and may lead to a change in smoking behaviour and variations in the efficacy of NRT (Nakajima et al 2000, Yildiz et al 2004, Siu & Tyndale 2007).

While CYP2A6 is found in large amounts in the liver, it has not been identified in the human brain. However, in the CNS (especially following induction by exposure to nicotine) human CYP2B6 may play a role in nicotine metabolism, at least in some brain regions. This could be a factor in the development of central tolerance to nicotine observed both in animal models and in human smokers (Miksys et al 2000).

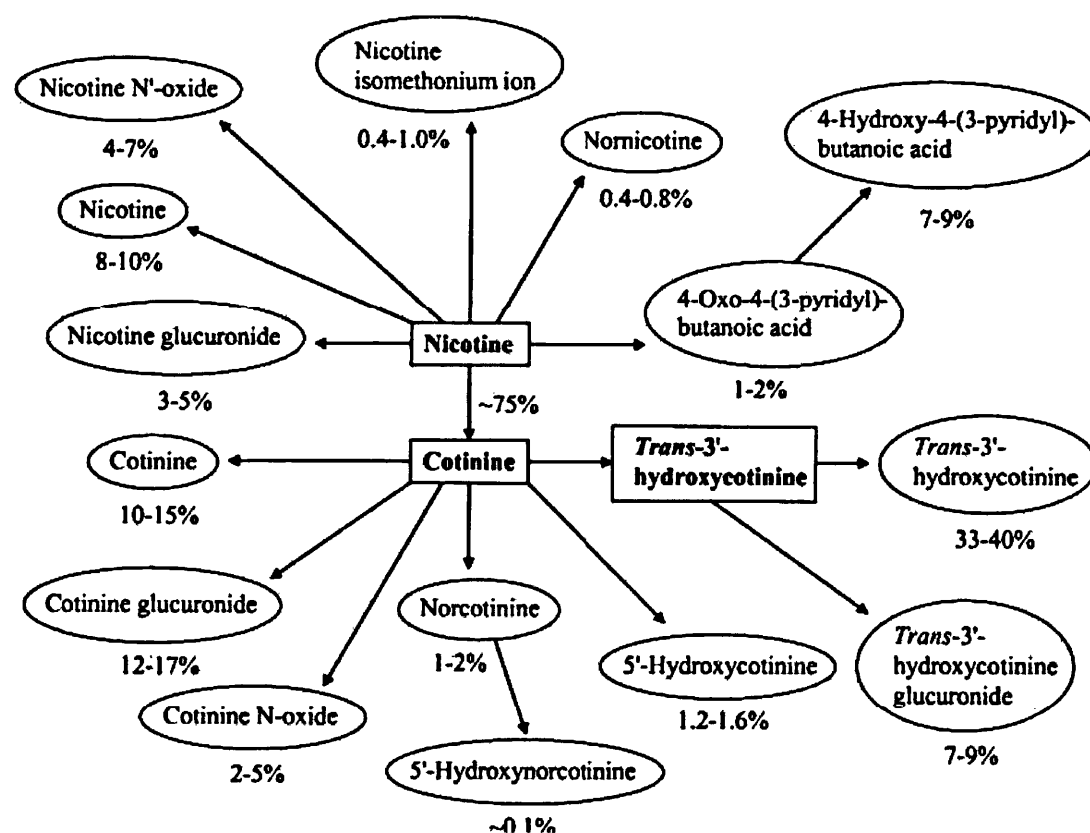
Compared to humans, the metabolism of nicotine to cotinine is less extensive in rats and nicotine-1'-N-oxide is the major nicotine metabolite in this species (Micu et al 2003, Tutka et al 2005). Nicotine is metabolised to cotinine primarily by hepatic CYP2B1, the rat homologue of CYP2B6, which has overlapping substrate specificity and is also found in other organs such as lung and brain (Miksys et al 2000, Micu et al 2003). Both CYP2E1 and 2B1/2 are expressed in various brain regions, including dopaminergic neurons in the substantia nigra where they may play a role in dopamine regulation. CYP2E1 expression has

also been detected in human brain regions, including hippocampus, medulla and substantia nigra (Howard et al 2003). Compared with the liver, however, the brain is generally a poor metabolising organ for nicotine biotransformation (Ghosheh et al 2001).

Members of the CYP2A and CYP2B families, along with CYP2E1, also bioactivate several procarcinogens, including tobacco-specific nitrosamines such as N-nitrosodimethylamine (NDMA) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), to their reactive intermediates. These are implicated in the development of tobacco-related cancers, which could be exacerbated if the production of such carcinogens is increased by the upregulation of CYPs due to chronic nicotine exposure (Miksys et al 2000, Howard et al 2001 & 2003, Iba et al 1999).

1.2.2 Metabolites of nicotine

Figure 1.2: Quantitative scheme of nicotine metabolism



Estimated average (urinary) excretion of nicotine metabolites as percent of total nicotine dose in man (from Hukkanen et al 2005)

In both rats and humans, the primary urinary metabolites are cotinine and secondary cotinine metabolites, together with variable but small amounts of nicotine-N-oxide, norcotinine, and nornicotine (Crooks & Dwoskin 1997). Figure 1.2 summarises the relative amount various metabolites contribute to the breakdown of the total nicotine dose administered.

1.2.2.1 Cotinine

In man, 70-80% of administered nicotine is converted to cotinine (Benowitz & Jacob 1994), making cotinine the major metabolite of nicotine. It has a much longer half-life (15-19 h) than the parent compound (2-3 h), which means that nicotine administration results in much higher blood concentrations of cotinine than nicotine (Benowitz et al 1983, Schneider et al 2001, Buccafusco & Terry 2003).

Cotinine has been reported to possess weak activity at nAChRs *in vitro*. Both cotinine and nicotine dose-dependently increase intracellular Ca^{2+} levels in bovine adrenal chromaffin cells, indicative of agonist activity, but pre-treatment with a low concentration of nicotine or cotinine was shown to inhibit the Ca^{2+} increase in response to the other compound (Vainio et al 1998a & 2000). Pre-treatment of these cells with cotinine for 10 min also dose-dependently decreased nicotine-evoked [^3H]noradrenaline release, but did not reduce potassium-evoked release (Vainio et al 1998b). Audesirk and Cabell have reported opposing effects of nicotine and cotinine in terms of cell survival and axon or dendrite branching in cultured hippocampal cells (Audesirk & Cabell 1999). On the other hand, cotinine has been observed to have cytoprotective effects similar to nicotine in PC12 cells (Buccafusco & Terry 2003).

Furthermore, nicotine appears to inhibit immune responses, whereas cotinine had no such effect (Geng et al 1995).

Cotinine can evoke DA release in brain slices in a concentration-dependent manner (EC_{50} 30 μM), although it is less potent than nicotine (EC_{50} 0.1-4.0 μM), and nornicotine (EC_{50} 1 μM) (Teng et al 1997, Dwoskin et al 1993 & 1999a). Nicotine-induced DA release in conscious rats also appears to be inhibited by prior intravenous administration of cotinine, although the dose used (500 $\mu\text{g/kg}$) was quite large (Sziraki et al 1999).

Evaluation of cotinine on aspects of human memory processing and on animal behaviour, such as operant food-motivated responding, has shown biphasic effects, with doses in the 0.1-0.5 mg/kg range providing a positive effect, and higher doses (1-30 mg/kg) resulting in reduced performance levels (Crooks et al 1997, Herzig et al 1998, Risner et al 1985).

1.2.2.2 *Trans*-3'-hydroxycotinine

In humans, (S)-Cotinine is metabolised primarily to (3'R,5'S)-*trans*-3'-hydroxycotinine (3-HC), with only very little conversion to the stereoisomer, (3'R,5'S)-*cis*-3'-hydroxycotinine, although the percentage appears to be higher in some animal species. Approximately 60-75% of 3-HC is excreted unchanged in the urine, with ~30% metabolised further and excreted as 3-HC glucuronide. The ratio of plasma 3-HC/cotinine appears to be inversely correlated with the plasma nicotine concentration during *ad libitum* cigarette smoking, suggesting this might be useful as a marker of CYP2A6 activity (Benowitz & Jacob 2001).

1.2.2.3 Norcotinine

Norcotinine has been shown to occur as a urinary metabolite of cotinine in dogs, mice, rats and humans, and is formed *in vitro* from cotinine in hepatic, pulmonary, and renal tissues (Ghosheh et al 1999 & 2001). Following nicotine administration, it can be detected in the urine of rats and humans, accounting for ~2% of the systemic nicotine dose in the latter, but was not observed after administration of cotinine to humans (Wada et al 1961, Bowman et al 1959, Tutka et al 2005). Norcotinine is also found in brain after peripheral nicotine administration, and has a half-life of approximately 4 hours. Brain norcotinine appears to originate from 5'-C-oxidation of brain nornicotine, rather than from N-demethylation of cotinine, as occurs peripherally (Ghosheh et al 2001). In their paper, Crooks et al (1997) refer to some unpublished data indicating that norcotinine neither evokes the release of [³H]dopamine from rat striatal slices, nor inhibits [³H]dopamine uptake into rat striatal synaptosomes, and there is no evidence to suggest that norcotinine is pharmacologically active, however, very little research on the actions of this nicotine metabolite has been conducted (Ghosheh et al 2001).

1.2.2.4 Nornicotine

Nornicotine is a major tobacco alkaloid, constituting 15-20% of total alkaloid content in tobacco (Dwoskin et al 2001). In addition, nornicotine is a very minor N-demethylated peripheral metabolite of nicotine in various animal species, with only ~8% (in rats) or ~1% (in humans) of peripherally administered nicotine converted to nornicotine in the periphery (Ghosheh et al 1999 & 2001, Crooks & Dwoskin 1997, Dwoskin et al 2001). The majority of nornicotine excreted by cigarette smokers is derived from metabolised nicotine, while up to 40% may be absorbed directly from tobacco (Tutka et al 2005). Although only a minor metabolite in the periphery, nornicotine is a major nicotine metabolite in the CNS and has a significantly longer half-life (7-8 h) than nicotine (1-2 h) (Dwoskin et al 1999b & 2001). Nicotine appears to be transformed to nornicotine locally in the brain, and the metabolite has been shown to accumulate in rat CNS to pharmacologically relevant concentrations following repeated peripheral nicotine administration. It seems likely that nornicotine may also reach pharmacologically relevant concentrations in smokers' brains as a result of direct exposure to nornicotine in tobacco, indirect exposure from nicotine metabolism, and due to the comparatively longer brain residence time of nornicotine, which results in CNS accumulation (Dwoskin et al 2001). Nornicotine has been shown to evoke the release of [³H]dopamine from rat striatal and nucleus accumbens slices in a concentration- and calcium-dependent manner (Dwoskin et al 1993, Green et al 2001), which is both mecamylamine- and dihydro-β-erythroidine-sensitive (Teng et al 1997), suggesting a nAChR-mediated mechanism of action. It competes with [³H]nicotine for high affinity binding sites in rat brain membranes, and is reported to have a 10-fold lower affinity for nicotinic receptors than nicotine. However, in DA release studies, nornicotine and nicotine were found to have similar potency (Crooks & Dwoskin 1997). Nornicotine has been reported to produce stimulant-like locomotor sensitisation effects (Dwoskin et al 1999b), which appear to be dependent upon activation of D₂ DA receptors in the mesolimbic system (Green et al 2002), as well as discriminative stimulus effects (Dwoskin et al 2001). This metabolite has also been observed to maintain self-administration behaviour (Bardo et al 1999) and decrease nicotine self-administration in rats (Dwoskin et al 2001), suggesting

that it may desensitise nAChRs. It is possible that nicotine self-administration behaviour in both animal models and smokers is maintained, at least in part, by prolonged nAChR stimulation by nicotine even after nicotine concentrations have diminished below the pharmacologically active range.

1.3 Sites of action – nicotinic acetylcholine receptors (nAChRs)

In order to understand nicotine addiction it is essential to have some knowledge of the structure and function of the nicotinic acetylcholine receptors, through which nicotine mediates its effects. nAChRs are found in the brain, autonomic ganglia, and the neuromuscular junction. Most relevant to nicotine addiction are the neuronal nAChRs, which are found throughout the brain, with the greatest number of binding sites in the cortex, thalamus, and interpeduncular nucleus, and further substantial binding in the amygdala, septum, brain stem motor nuclei and locus coeruleus (Gotti & Clementi 2004).

1.3.1 Structure of nAChRs

Langley's description of a "nicotinic receptive substance" at the neuromuscular junction (Langley 1907) led to the formulation of the receptor concept and kick-started research into the structure and function of nAChRs. Along with GABA_A, glycine and 5HT₃ receptors, nAChRs belong to the superfamily of ligand-gated ion channels. Each nAChR subunit has 4 transmembrane domains, and subunits assemble to form pentameric structures arranged around a central ion-conducting channel, which opens upon binding of acetylcholine (ACh; Karlin 2002, Gotti & Clementi 2004). Distinct nAChR subtypes are found at the neuromuscular endplate, the sympathetic and parasympathetic ganglia, and in the central nervous system (CNS) of vertebrate species, where they regulate cholinergic transmission, cell excitability, and muscle contraction, as well as modulating the release of other neurotransmitters such as dopamine (DA), noradrenalin (NA), γ -aminobutyric acid (GABA) and glutamate. As a result, they play an important role in physiological functions such as arousal, sleep, fatigue, anxiety, the central processing of pain, food intake, and a variety of cognitive functions (Karlin 2002, Laviolette & van der Kooy 2004, Nicke et al 2004, Gotti & Clementi 2004).

Twelve neuronal nAChR subunits ($\alpha 2$ - $\alpha 10$, $\beta 2$ - $\beta 4$) have been identified in vertebrates, although a mammalian homologue of the avian $\alpha 8$ subunit has not yet been found. Embryonic vertebrate muscle and Torpedo nAChR (see Figure 1.4) on the other hand are composed of $\alpha 1$ and $\beta 1$, as well as γ and δ subunits, with ϵ replacing the γ subunit in adult muscle (Karlin 2002, Laviolette & van der Kooy 2004, Gotti & Clementi 2004).

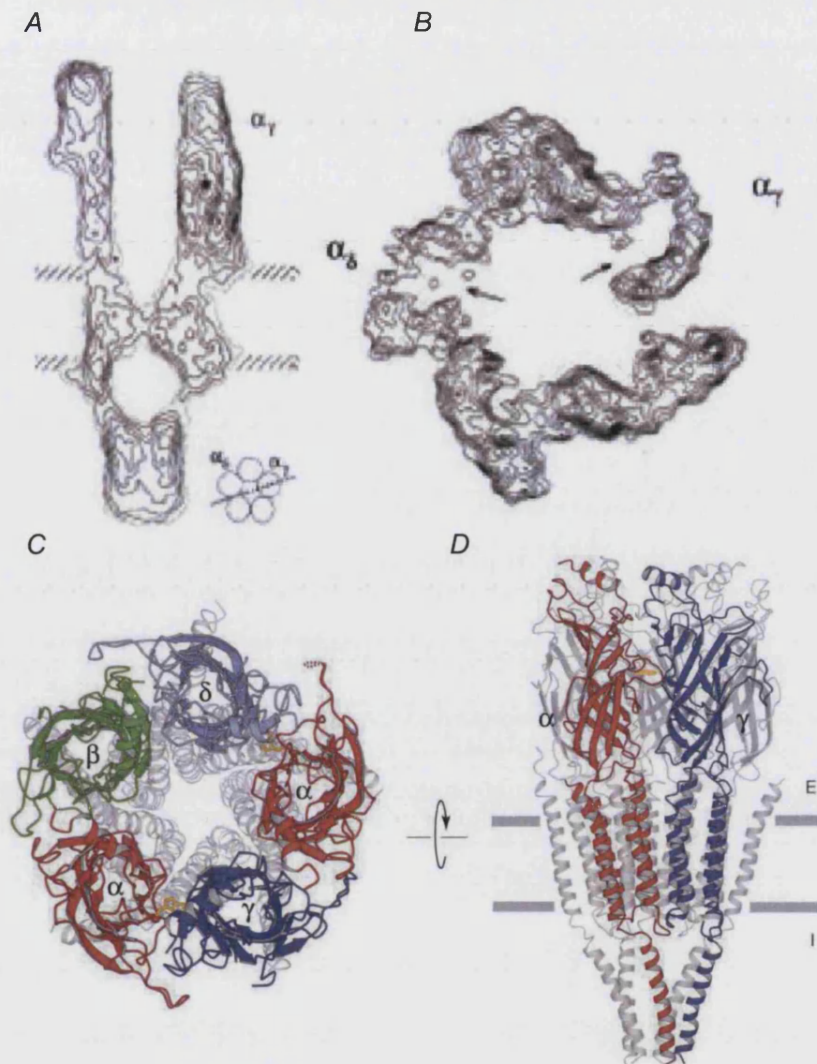
The $\alpha 7$ - $\alpha 9$ subunits can form homomeric nAChRs, whereas the remaining subunits form different combinations of α - and β -subunits, with $\alpha 4\beta 2^*$ nAChRs being the most predominant subtype (Gotti & Clementi 2004). The $\alpha 2$, $\alpha 3$, $\alpha 4$ and $\alpha 6$ subunits require co-expression of at least one β subunit ($\beta 2$ or $\beta 4$) to form functional channels (Nicke et al 2004).

Figure 1.3: Subtypes of nAChR found in the VTA and striatum

| Brain region | nAChR subtypes | Neuronal type |
|--------------|---|---------------|
| VTA | $(\alpha 4)_2(\beta 2)_3$, $(\alpha 7)_5$ | GABAergic |
| | $\alpha 4\alpha 6\alpha 5/\beta 3(\beta 2)_2$, $(\alpha 4)_2\alpha 5(\beta 2)_2$, $(\alpha 7)_5$ | Dopaminergic |
| Striatum | $(\alpha 4)_2(\beta 2)_3$, $(\alpha 4)_2\alpha 5(\beta 2)_2$, $(\alpha 6)_2(\beta 2)_2\beta 3$, $\alpha 4\alpha 6(\beta 2)_2\beta 3$ | Dopaminergic |

Summary of somatodendritic nAChR subtypes proposed to reside on dopaminergic and GABAergic neurones in the VTA and presynaptic nAChRs on dopaminergic boutons in the terminal field (from Wonnacott et al 2005)

The mesocorticolimbic dopamine system is central to reward and reinforcement of both natural rewards and addictive drugs, such as nicotine (see section 1.4). Neurons involved in this "reward circuitry" express nAChRs consisting of a variety of subunit combinations (see Figure 1.3). These subtypes are thought to have a stoichiometry of $(\alpha)_2(\beta)_3$ or $(\alpha)_3(\beta)_2$ (Gotti & Clementi 2004, Wonnacott et al 2005). The areas of distribution for various subunits, and thus also subunit combinations, appear to overlap, resulting in a great diversity of nAChRs, with distinct pharmacological, physiological and electrical properties, depending on the subunit composition of the channel (Gotti & Clementi 2004, Wonnacott et al 2005).

Figure 1.4: Structure of nicotinic acetylcholine receptors

Electron microscopic images (a & b; averaged structure) and ribbon diagrams (c & d) of nAChR derived from tubular crystals of *Torpedo* postsynaptic membranes (a & b from Miyazawa et al 1999, c & d from Unwin 2005)

a) Cross-section along the central axis of the receptor, illustrating the position of the ion channel within the membrane (hatched lines). The asterisk in the extracellular portion of the receptor indicates the proposed acetylcholine (ACh) binding site, with the dotted line highlighting the narrow tunnel connecting the ACh binding pocket to the vestibule of the ion channel

b) Cross-section parallel to the membrane plane, showing the pentameric arrangement of receptor subunits, with the putative ACh binding pockets indicated by arrows

c) Ribbon diagram of the nAChR seen from the synaptic cleft

d) Ribbon diagram of the nAChR as viewed parallel with the membrane plane (the front 2 subunits are highlighted for clarity; E = extracellular, I = intracellular)

Most of the work on structural characterisation of nAChRs has been done using the electric organs of the electric ray (*Torpedo*) and eel (*Electrophorus*), which provide a rich source of nAChRs. High-resolution images (4 Å), obtained using electron microscopy techniques (Unwin 2005), have provided valuable information about the channel architecture and dimensions (see Figure 1.4), with crystallisation studies of nAChRs and acetylcholine binding protein (AChBP) giving a deeper insight into the molecular structure (Brejc et al 2001, Miyazawa et al 2003).

AChBP is a homopentameric, soluble protein secreted by snail glial cells into cholinergic synapses where it modulates synaptic transmission by binding acetylcholine (ACh). It is a structural and functional homologue of the extracellular portion of nAChRs (especially $\alpha 7$ homomeric receptors), which contains the amino-terminal ligand-binding domain (Brejc et al 2001, Miyazawa et al 2003, Unwin 2005).

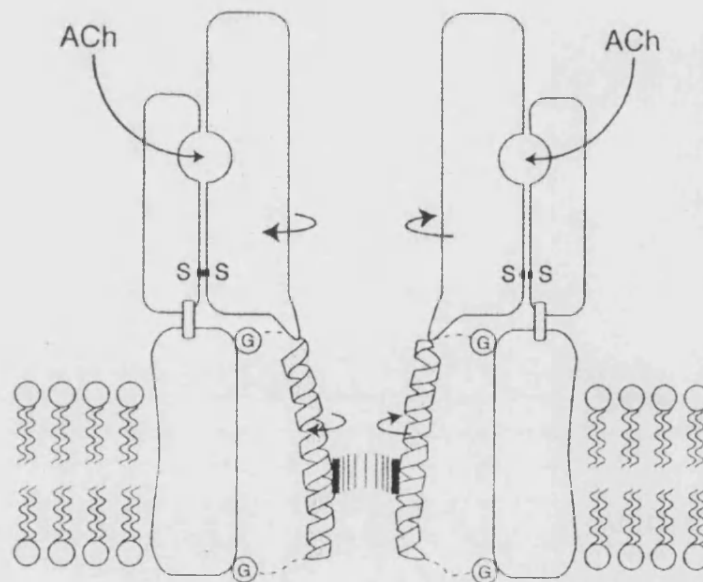
The receptor channel is funnel-shaped, with a narrow section towards the cytoplasmic end of the membrane-spanning pore, which selects for ion charge and size, and determines conductance. The pore is composed of an inner ring of 5 α -helices, curving radially to create a tapering path for the ions, and an outer ring of 15 α -helices, which coil around each other and shield the inner ring. This region also contains the "gate", a constricting hydrophobic girdle near the middle of the lipid bilayer formed by weak interactions between neighbouring inner helices (Karlin 2002, Sine 2002, Miyazawa et al 2003, Unwin 2005).

The ACh and nicotine binding sites are located at the interface between the α subunit and either an adjacent β subunit (heteromeric neuronal nAChR), another α subunit (homomeric nAChR), or, in the case of muscle nAChRs, a δ , γ or ϵ subunit. The $\alpha 5$, $\beta 1$ and $\beta 3$ subunits appear to play a more structural role, possibly also modulating channel function and influencing membrane transport and targeting of nAChRs, while the remaining subunits can contribute to the ACh binding site (Karlin 2002, Nicke et al 2004, Gotti & Clementi 2004).

1.3.2 Function of nAChRs

nAChRs contain multiple agonist- and non-competitive-antagonist binding sites, as well as a gate, all of which interact at a distance through changes in the quaternary structure of the receptor to modulate its function (Karlin 2002, Sine 2002). When an agonist, such as acetylcholine or nicotine, enters the ligand-binding domain, it triggers rotations of the protein chains on opposite sides of the entrance to the pore (see Figure 1.5). These rotations are communicated through the inner helices, allowing the channel gate to open, and permitting the passage of cations (Na^+ , K^+ , and Ca^{2+}) through the channel pore (Miyazawa et al 2003, Laviolette & van der Kooy 2004, Unwin 2005). In neurons, activation of nAChRs is involved in calcium homeostasis and signalling, both via an influx of Ca^{2+} through the nAChR channel, but also because nAChR-mediated depolarisation of the plasma membrane can activate voltage-operated calcium channels (VOCCs), leading to an increase in intracellular Ca^{2+} , which can induce further mobilisation of Ca^{2+} from intracellular stores (Gotti & Clementi 2004).

Figure 1.5: Proposed model for nAChR gating mechanism



ACh binding to the receptor induces rotations in the alpha-subunits, which are transmitted to the gate through the M2 helices (coils lining the pore). This destabilises the gate and causes the helices to adopt an alternative configuration that is permeable to the ions. S-S: disulphide bridge, G: glycine residue (from Miyazawa et al 2003).

Muscle nAChRs, located at the neuromuscular endplate, transmit the nerve stimulus to the muscle, by causing depolarisation via the influx of cations through the opening receptor channels. This leads to contraction of the muscle fibres (Ganong 1999).

Neuronal nAChRs appear to be located at the cell body, as well as on neuronal processes, both pre- and postsynaptically (Laviolette & van der Kooy 2004).

Postsynaptic nAChRs are involved in the generation of action potentials by enabling the depolarisation of the postsynaptic neuron, again via cation influx through the open ion channels. Presynaptic nAChRs directly enhance the Ca^{2+} -dependent release of many neurotransmitters, as well as modulating dopamine release indirectly by regulating glutamate and γ -aminobutyric acid (GABA) release. nAChRs located presynaptically can also influence synaptic efficacy and long-term potentiation or long-term depression (Mansvelder & McGehee 2000, Wonnacott et al 2005).

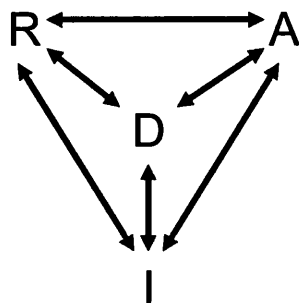
The nAChR receptor complex can exist in several conformational states (see Figure 1.6), which are dynamically regulated by exposure to the agonist: closed, open and desensitised (Karlin 2002, Sine 2002, Laviolette & van der Kooy 2004, Unwin 2005). When nAChRs are exposed to an agonist repeatedly or for prolonged periods, they enter an inactive, desensitised state, from which they can recover upon agonist removal. The recovery time appears to depend on the duration of agonist exposure, and the agonist used, as well as the receptor subtype (Grady et al 1994, Reitstetter et al 1999). A reasonable explanation might be that during longer exposure to an agonist nAChRs can enter deeper levels of desensitisation (Pidoplichko et al 1997, Reitstetter et al 1999).

Homomeric $\alpha 7$ receptors appear to have more rapid activation and desensitisation kinetics than $\alpha 4\beta 2$ high-affinity nAChRs, which take much longer to recover from the desensitised state (Wooltorton et al 2003).

Nicotine addiction is characterised by repeated nicotine intake, resulting in sustained levels of nicotine in the blood. As a consequence of such prolonged exposure to nicotine, nAChRs in the brain are likely to be desensitised for long periods of time (most of the day in a smoker). Chronic exposure to nicotine, or other nAChR agonists, via subcutaneous infusion or repeated injections, has been observed to elicit an increase in the number of nicotine binding sites in the brains of rodents (Wonnacott 1990, Flores et al 1992, Parker et al 2004). The

$\alpha 4\beta 2^*$ nAChR displays the highest level of upregulation, with relatively little effect on other receptor subtypes (Nguyen et al 2003). Numbers of [3 H]nicotine binding sites in the post-mortem brains of smokers are also higher than in non-smokers (Benwell et al 1988). This observed increase in binding appears to be due to an increase in receptor density, rather than affinity (Marks et al 1985 & 1987, Schwartz & Kellar 1985).

Figure 1.6: Conformational states of nAChRs



Upon agonist binding, nAChRs undergo an allosteric transition from the closed, resting conformation to an open state that conducts cations (sodium, potassium, calcium). In the active/open conformation, the nAChR binds agonists with low affinity. The continued presence of agonist leads to ion channel closure and receptor desensitisation. Prolonged agonist exposure may produce an inactivation state, from which recovery is very slow.

*R = low-affinity resting state; A = active/open receptor state; D & I desensitised states.
(adapted from Changeux & Edelstein 1998)*

Because nAChRs are subject to profound and prolonged desensitisation upon exposure to agonists, it was initially suggested that this might underlie the mechanism of agonist-induced upregulation (Marks et al 1985, Schwartz & Kellar 1985). However, desensitisation alone is not sufficient to induce measurable upregulation of binding sites. Treatment with nicotine concentrations sufficient to achieve complete desensitisation of receptors does not appear to result in upregulation (Rowell & Li 1997, Marks et al 1994). A single injection of nicotine, which should desensitise the receptors, is without effect on receptor numbers (Schwartz & Kellar 1985), instead, repeated daily injections or chronic infusion appear to be required for upregulation. Increased receptor numbers are observed after several days (Schwartz & Kellar 1985, Marks et al 1985), compatible with altered protein turnover, which could result from increased receptor synthesis or assembly, increased incorporation into the membrane from a "reserve" pool of receptors, or decreased degradation. However, the absence of an increase in mRNA points to a post-transcriptional

mechanism. Upon termination of drug treatment, recovery to the original receptor density occurs over a similar time-course (Wonnacott 1990). Surface-expressed nAChRs are constitutively and rapidly endocytosed from the plasma membrane and directed into a lysosomal degradation pathway. Under normal conditions, surface nAChRs are constantly replaced by new receptors from the secretory pathway. Chronic exposure of surface nAChRs to nicotinic agonists is sufficient for induction of surface receptor upregulation but does not appear to alter the endocytic trafficking or subunit mRNA levels (Sallette et al 2005). It has been proposed that an internal pool of immature nAChR subunits may reside in the endoplasmic reticulum (ER), where they are thought to oligomerise prior to the assembly of mature pentameric receptors. This would help explain the observed accumulation of high-affinity binding sites in intracellular compartments (Harkness & Millar 2002, Whiteaker et al 1998, Sallette et al 2005). The immature receptor components may contain exposed ER retention signals, which act to keep unassembled components in the ER until packaging is complete (Nashmi et al 2003, Ren et al 2005), as appears to be the case with muscle nAChRs (Keller et al 2001). Nicotine could bind to these precursors to stabilise a conformation possessing higher affinity for nicotine and facilitate progression to further steps of maturation toward high-affinity receptors, such as packaging into secretory vesicles and exit from the ER (Darsow et al 2005, Sallette et al 2004 & 2005).

1.4 Addiction & reward

Several neural circuits in the brain are thought to mediate the addictive actions of drugs of abuse, including nicotine. These brain structures represent reward pathways, which are very old from an evolutionary point of view and presumably evolved to mediate an individual's response to natural rewards such as food, sex and social interaction (Wise 1996, Nestler 2002).

1.4.1 Reward circuitry

Nicotine induces feelings of pleasure and reward in humans and other species, but like many other addictive drugs it also has aversive, unpleasant effects. Many people experience noxious effects (nausea, coughing, dizziness) on their initial experience with tobacco, although tolerance to these aversive effects develops with repeated exposure (Laviolette & van der Kooy 2004).

Understanding how the neural systems interact may yield important clues about how the brain responds to acute nicotine exposure, and how continued exposure can lead to dependence.

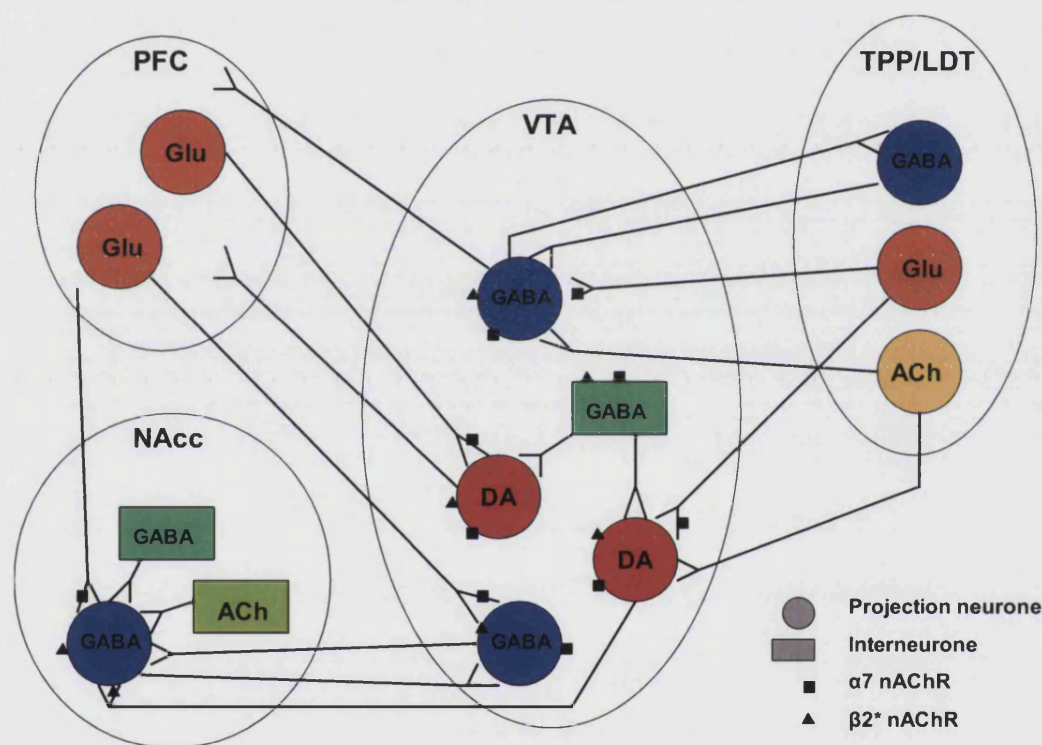
The mesocorticolimbic dopamine (DA) system (see Figure 1.7) has been implicated in the rewarding effects of a variety of addictive drugs, including cocaine, alcohol, opiates and nicotine (Wise 1996, Corrigal 1999, Gerrits et al 2002, Nestler 2002, Wonnacott et al 2005). Cell bodies of this system originate in the ventral tegmental area (VTA) and project to the forebrain, mainly the nucleus accumbens (NAcc), olfactory tubercle, (pre-) frontal cortex, amygdala and septal areas. Functional interactions between DA, GABA and glutamate within the VTA appear to play a vital role in mediating the motivational properties of nicotine (Koob 1992, Wise 1996, Pidoplichko et al 1997 & 2004, Laviolette & van der Kooy 2004, Wonnacott et al 2005).

VTA GABA neurons provide inhibitory input to the mesocorticolimbic DA neurons (Koob 1992, Wise 1996, Laviolette & van der Kooy 2004, Wonnacott et al 2005), and project to the brainstem mesopontine region, including the tegmental pedunculopontine nucleus (TPP). The VTA also receives excitatory glutamatergic inputs from several cortical and subcortical structures (Pidoplichko et al 1997 & 2004), as well as cholinergic inputs from the TPP and adjacent laterodorsal tegmental nucleus (LDT), and inhibitory GABA inputs from the TPP (Koob 1992, Wise 1996, Laviolette & van der Kooy 2004, Wonnacott et al 2005).

Electrical stimulation of the TPP elicits striatal DA efflux, whereas LDT stimulation leads to a similar DA efflux in the NAcc through activation of cholinergic and glutamatergic receptors in the VTA (Wise 1996). The importance of these brain areas in relation to the effects of nicotine has been demonstrated by means of lesion studies. LDT lesioned rats showed no differences in responding to nicotine compared to saline, whereas in sham-

lesioned control rats an initial depression of locomotor activity was observed followed by a progressive increase in locomotion upon repeated exposure (Alderson et al 2005). The spontaneous locomotor activity of lesioned rats was also decreased compared to controls. Intravenous self-administration (see Chapter 4) was significantly increased following lesions of the posterior TPP compared to sham-lesioned controls, however lesions of the anterior TPP had no significant effect (Alderson et al 2006).

Figure 1.7: Simplified schematic of afferent and efferent connections between the VTA, NAcc, PFC and TPP/LDT



Major pathways using dopamine (DA), GABA, acetylcholine (ACh) and glutamate (Glu), and locations of $\alpha 7$ - and $\beta 2^*$ nAChRs are illustrated. See text for details and abbreviations (adapted from Wonnacott et al 2005).

Nicotine acts through nAChRs, enhancing DA release (Stolerman & Shoaib 1991, Pidoplichko et al 2004). *In vitro* (transmitter release assays) and *in vivo* (microdialysis, voltammetry) techniques (discussed in more detail in Chapter 2) have shown that administration of nicotine, at concentrations within the physiological range of plasma levels obtained by smokers ($\sim 0.5 \mu\text{M}$), potently activates DA neurons in the VTA (Pidoplichko et al 1997 & 2004) and increases

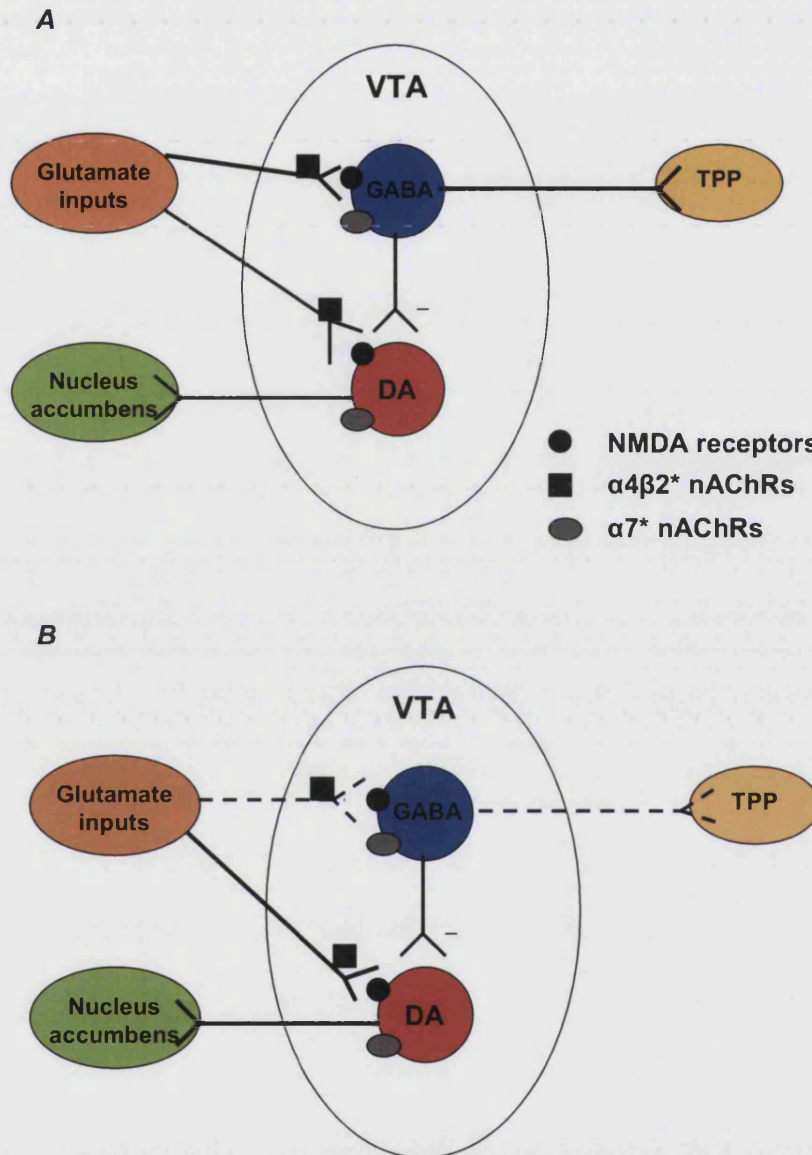
DA release in the NAcc (Stolerman & Shoaib 1991, Wise 1996, Corrigall 1999, Gerrits et al 2002, Nestler 2002, Wonnacott et al 2005). This activation is followed by desensitisation of nAChRs with continued exposure to nicotine (Pidoplichko et al 1997 & 2004), suggesting that acute excitatory action of nicotine on DA neurons might signal its reinforcing, rewarding effect, whereas the long-lasting desensitisation of VTA nAChRs may represent a cellular basis of nicotine tolerance. This could explain why smokers tend to enjoy the first cigarette of the day most, as overnight abstinence would have allowed nAChRs to recover from their state of prolonged desensitisation (Laviolette & van der Kooy 2004).

In rat and mouse striatum, two principal nAChR populations have been identified using various strains of knockout mice (Zoli et al 1998, Marubio & Changeux 2000, Whiteaker et al 2002, Champtiaux et al 2003, Salminen et al 2004, Gotti & Clementi 2004): one population contains the $\alpha 4$ and $\beta 2$ subunits but not $\alpha 6$ ($\alpha 4\beta 2^*$), while the other contains $\alpha 6$ and $\beta 2$ subunits ($\alpha 6\beta 2^*$). These populations account for approximately 70 and 20% of striatal nAChRs, respectively.

There is evidence to suggest that $\alpha 7$ nAChRs play a role in the regulation of glutamate release (Pidoplichko et al 2004). Lesions of the prefrontal cortex (PFC), a region providing glutamatergic inputs to the VTA, reduce binding of the $\alpha 7$ antagonist α -bungarotoxin in the VTA, and blockade of NMDA (N-methyl-D-aspartate) or $\alpha 7$ receptors in the VTA reduces the increase in mesolimbic DA release induced by nicotine (Schilström et al 1998 & 2000, Laviolette & van der Kooy 2004).

In the acute stage of nicotine reward signalling, activation of GABA neurons in the VTA by nicotine produces rewarding effects through a GABA-dependent system projecting to the TPP (Laviolette et al 2002), as illustrated in Figure 1.8 a. These effects are thought to involve activation of $\alpha 7$ presynaptic nAChRs, however, nicotine may also exert its motivational effects through direct actions on $\beta 2^*$ nAChRs located on GABA or DA neurons, since pharmacological blockade or genetic deletion of this subunit appear to block both aversive and rewarding effects of nicotine (Pidoplichko et al 1997 & 2004, Picciotto et al 1998, Klink et al 2001, Wooltorton et al 2003, Marubio et al 2003).

Figure 1.8: Reward signalling in the VTA with acute and chronic nicotine exposure



a) In the acute stage, activation of dopaminergic neurons by nicotine induces aversive effects, whereas GABA neurons and associated inputs to the TPP mediate rewarding effects.

b) Repeated nicotine exposure leads to desensitisation of the GABA system and results in sensitisation of dopaminergic pathways, which signal the incentive salience (ie how much the drug is craved).

[from Laviolette & van der Kooy 2004; GABA = γ -aminobutyric acid; NMDA = N-methyl-D-aspartate; TPP = tegmental pedunculo pontine nucleus; VTA = ventral tegmental area]

It has been proposed that, with repeated nicotine exposure (see Figure 1.8 b), the GABA system becomes desensitised (Pidoplichko et al 2004), leading to a net shift towards DA neuronal activity due to reduced inhibition by GABA neurons (Wise 1996, Laviolette & van der Kooy 2004). The desensitisation of inhibitory inputs to the DA system also correlates with enhanced glutamatergic input to DA neurons (Pidoplichko et al 2004) through nicotine acting on presynaptic α_7 nAChRs on VTA glutamatergic terminals (Nomikos et al 2000), which show a lesser degree of desensitisation. This shift in the functional balance between GABA and DA neuronal populations may lead to a dysregulated DA signal in the VTA, resulting in the aversive psychological effects of nicotine craving and withdrawal, and/or the potentiation of the incentive salience of nicotine and its compulsive use (Laviolette & van der Kooy 2004).

Nicotine has also been shown to suppress striatal dopamine release during non-reward low firing frequencies through nAChR desensitisation, while selectively enhancing reward-related dopamine release by relieving short-term depression at higher burst-like firing frequencies (Rice & Cragg 2004). This mechanism is likely to play an important role in the ability of nicotine to enhance the reinforcing efficacy of reward-related stimuli (Caggiula et al 2001), including non-nicotine conditioned stimuli (e.g. visual cues), which can support nicotine self-administration behaviour (see Chapter 4). This exaggeration of dopaminergic reinforcement mechanisms results in a powerful signal for nicotine-associated behaviours, habit and addiction at a synaptic level (Schultz 2002).

1.4.2 Addiction

Addiction can be defined as the loss of control over drug use, or the compulsive seeking and taking of drug regardless of the consequences (Nestler 2002). The main factor common to all drug addictions is drug-seeking behaviour, and the ability to act as a positive reinforcer is the minimum requirement for maintaining drug-seeking behaviour. Drugs of abuse are very powerful reinforcers and can motivate high rates of operant responding even in conditions of limited access (where the subject is not dependent), and drugs readily self-administered

intravenously (see Chapter 4) or orally by animals appear to correspond well with those of high abuse potential in humans. However, a drug serving as a positive reinforcer under certain circumstances may be ineffective in other conditions; drug dose, genetic factors, social circumstances and previous history of the individual are also important factors in determining reinforcing effectiveness (Stolerman 1992, Koob 1992). Environmental stimuli and rituals associated with drug taking behaviour (e.g. manual manipulation of cigarette) can also provide reinforcement and lead to relapse in an abstinent drug user (Le Foll & Goldberg 2005).

In a dependent subject, the termination or prevention of aversive withdrawal symptoms may constitute a source of negative reinforcement (Wise 1996). It is thought that environmental stressors may enhance signs of nicotine withdrawal, and that nicotine encourages adaptive changes in the brain, which facilitate coping with stress and anxiety (Stolerman & Shoaib 1991, Stolerman 1992, Koob 1992, Nestler 2002).

The strength of drug-seeking behaviour is seen as the result of a drug's positive reinforcing and discriminative stimulus properties. However, the ability of a drug to serve as a discriminative stimulus is not, by itself, sufficient to indicate that it will support drug-seeking behaviour. Almost all addictive drugs have discriminative stimulus effects, including psychomotor stimulants, anxiolytics and sedative/hypnotics, opioids, ethanol and nicotine (Stolerman 1992).

Discriminative effects may directly promote drug-seeking behaviour due to previous association of such behaviour with the perceived effects of the drug (Nestler 2002). These "priming" effects are distinct from a drug's reinforcing actions, since they occur with passively administered instead of self-administered drugs, and appear to show pharmacological specificity: cocaine primes cocaine but not heroin self-administration, and vice versa. In contrast, self-administration behaviour maintained by a drug from one pharmacological class often persists when a reinforcing substance from another class is substituted (Stolerman 1992). Wild-type mice trained to self-administer cocaine maintained specific operant responding when the drug infusion was switched to nicotine, whereas in mutant mice lacking the $\beta 2$ nAChR subunit a reduction in responding was observed over the nicotine substitution period (Picciotto et al 1998). Knockout mice were also less able to discriminate between active (drug-

associated) and inactive levers (see Chapter 4 for more detail on drug discrimination and self-administration procedures).

Withdrawal from chronic treatment is also often associated with clear discriminative stimulus effects. The ability to perceive and identify the characteristic subjective effects of a drug and its withdrawal symptoms may therefore mediate drug-seeking, by indicating the effects and potency of a substance and directing the organism towards one substance rather than another (Stolerman 1992).

In addition, the ability to produce changes in mood is recognised as an important factor in many addictions, and is not limited to the production of euphoria; subjective effects of drugs are often described in terms of reduction of negative mood states such as anxiety, or as a means for escaping from an unendurable reality. Nicotine can produce a subjective state of euphoria resembling the effects of classical addictive drugs, although the intensity may not be sufficient to make this the sole or major source of reinforcement for smoking (Stolerman 1992).

Furthermore, there is the ability of drugs to provide a resource for users in terms of perceived improvement in performance. This has been applied to nicotine in particular, which appears to enhance accuracy during tests of sustained attention and improve the ability to process information, which is presented very rapidly (Stolerman 1992).

Environmental stimuli can become associated with the effects of drug (or drug withdrawal), leading to previously neutral stimuli acquiring secondary reinforcing properties following association with reinforcing doses of drugs (Berridge & Robinson 2003). In tobacco smoking, sensory stimuli such as the sight, taste and smell of cigarette smoke exert powerful control over usage, possibly due to the numerous previous pairings of such stimuli with the effects of nicotine, the primary reinforcer (Le Foll & Goldberg 2005).

Conditioned stimuli have also been implicated in the development of tolerance (and sensitisation) to effects of several drugs (opiates, alcohol, amphetamine and cocaine). In many studies, tolerance and sensitisation appear to be situation-specific, with a greater development of tolerance (or sensitisation) observed when a drug is tested in an environment where it was administered previously than in a distinctly different environment (Stolerman 1992, Rose et al

2000, Caggiula et al 2001). Attempts to treat nicotine addiction should therefore include not just pharmacotherapy to combat the reinforcing effects of nicotine, but also behavioural or psychotherapy to help deal with the conditioned stimuli associated with smoking (see section 1.6).

1.5 Tobacco consumption

Nicotine is the main psychoactive drug delivered by tobacco, producing most of the immediate pharmacological effects, including the addictive effects on brain and behaviour (Stolerman et al 1991). It is a peripheral vasoconstrictor and sympathetic stimulant, which probably has a weak causal role in some cardiovascular diseases (Foulds et al 2004).

Tobacco is available in the form of a variety of products, which can be divided into two categories: smoked tobacco and smokeless tobacco products.

1.5.1 Smoked tobacco

There are several forms of smoked tobacco, of which cigarettes are the most prevalent. Other forms include cigars, cigarillos and pipe tobacco. Tobacco smoke consists of volatile and particulate phases containing thousands of different substances, most of which are generated in the combustion process. Carbon monoxide gas preferentially binds to haemoglobin, thus reducing the oxygen-carrying capacity of the blood. "Tar" from tobacco smoke is a black sticky cocktail of chemicals containing more than 40 known carcinogens, which lodges in the lungs and upper airways and causes respiratory diseases by interfering with the normal functions of the airways (Stolerman et al 1991, Foulds et al 2004). Stated tar and nicotine yields are based on the way that machines, not people, smoke cigarettes. Smokers addicted to nicotine adjust their smoking (consciously and/or subconsciously) in order to achieve the desired nicotine intake and thereby avoid nicotine withdrawal. Even if a cigarette has a low nicotine yield, a smoker can extract a greater percentage of nicotine from each cigarette by taking deeper and longer inhalations, holding the smoke in the lung, and covering the perforations around the filter (Moxham 2000).

The cigarette is a very efficient device, which rapidly delivers the optimum dose of nicotine to the brain (Moxham 2000). Nicotine (typically 1-3 mg per cigarette) is absorbed from cigarette smoke, enters the arterial circulation, and is quickly distributed to body tissues (Benowitz 1996, Foulds et al 2004). It takes only 10-20 s for nicotine to pass through the brain. Nicotine levels then fall, owing to uptake by peripheral tissues and elimination of nicotine from the body.

Arteriovenous differences during cigarette smoking are substantial, with arterial levels exceeding venous levels six- to ten-fold. Rapid delivery of nicotine results in a more intense pharmacological response, due to higher arterial levels entering the brain and effects occurring rapidly, before there is adequate time for the development of tolerance in the naive smoker. Reinforcement of drug seeking is therefore immediate and is associated with the sensory, ritual and psychological effects of smoking (Schneider et al 2001). Nicotine levels in the brain decline between cigarettes, providing an opportunity for re-sensitisation of receptors so that positive reinforcement can to some extent occur with successive cigarettes despite the development of tolerance (Benowitz 1996). It is thought that in chronic smokers, on the other hand, tobacco consumption is maintained in order to avoid the withdrawal effects associated with nicotine abstinence (see section 4.5).

It is now accepted that tobacco smoking is highly addictive and that nicotine is the principal psychoactive component of tobacco smoke responsible for tobacco dependence. Tobacco smoking is the most prevalent form of drug abuse, and the treatment of tobacco dependence has been made a public health priority (US Dept of Health & Human Services 1989).

1.5.2 Smokeless tobacco products

Smokeless tobacco was the dominant form of tobacco until early in the 20th century when developments in tobacco cultivation, curing and manufacturing, along with the invention of the safety match, resulted in the increased popularity of cigarettes. Smokeless tobacco (ST) products are not burnt; instead they are placed in the cheek or between the lip and gum. ST is used in many countries around the world, including the Middle East and the Indian subcontinent, and is especially popular in Sweden (Rodu & Godshall 2006).

Most of the medical and epidemiological evidence shows that ST users do not have elevated risks for cardiovascular disease. In distinct contrast to smokers, ST users do not exhibit any significant differences compared to non-smokers with regard to heart rate, blood pressure, cardiac output and maximal working capacity, or levels of haemoglobin, hematocrit and leukocytes. ST users also do not appear to develop smoking-associated vascular changes, such as increased thickness of blood vessels and atherosclerotic plaques, and have no or only a modestly increased risk for heart attacks or strokes. They also have a much lower incidence of oral cancer than smokers (Huhtasaari et al 1992, Accortt et al 2002, Hergens et al 2005, Rodu & Godshall 2006).

Oral leukoplakias ("white plaque", a thickening of the mouth lining) occur in up to 60% of ST users at the site of ST use and are largely a result of local irritation. Dysplasia (a pre-cancerous stage) is seen only infrequently in ST leukoplakias (< 3%), whereas in leukoplakias due to smoking, dysplasia is observed in ~20% of cases. Although oral leukoplakias occur commonly in ST users, they primarily present irritation and only very rarely progress to oral cancer (Silverman et al 1984, Kaugars et al 1989, Grady et al 1990, Rodu & Godshall 2006).

Widespread use of ST has been alleged to serve as a gateway to smoking. However data from studies in the US and Sweden do not support this. In fact the opposite appears to be the case among Swedish men, with snus serving as a pathway away from smoking (Foulds et al 2003, Bates et al 2003, Rodu & Godshall 2006).

Three main types of ST are described in more detail below:

Powdered dry snuff

Dry snuff is made from fermented, fire-cured tobacco, which is then pulverised. Nasal inhalation of dry snuff was widely practiced in Europe in the 17th and 18th centuries but declined thereafter. Manufacturers now provide an array of flavoured dry snuff products but on the whole the use of dry snuff is declining (Rodu & Godshall 2006).

Loose leaf chewing tobacco

Loose-leaf chewing tobacco consists of air-cured leaf tobacco, which is shredded, coated with sweet flavouring solutions and then packaged. Chewing tobacco is typically used in large volumes, resulting in a golf ball-sized bulge in the user's cheek and large quantities of saliva, which users usually spit out. Consequently, the popularity of this product has waned, with consumption declining steadily over the past century (Rodu & Godshall 2006).

Moist snuff & "snus"

Moist snuff is currently the most popular form of ST in the US and consists of fire-and air-cured dark tobaccos, which are finely ground or cut. The user usually compresses a "pinch" between the thumb and forefinger, and places it inside the lip. Much less bulky than loose leaf chewing tobacco, moist snuff produces less saliva, but spitting is still common (Rodu & Godshall 2006). There is a long tradition of moist snuff use in Scandinavia, especially in Sweden, where "snus" is essentially the only type of ST product in use. Traditional American products undergo fermentation, which imparts characteristic flavours but also results in higher concentrations of unwanted bacterially mediated by-products (tobacco specific nitrosamines (TSNAs) and nitrite). In Sweden, moist snuff is subjected to a heat treatment similar to pasteurisation during manufacturing, yielding virtually sterile products containing only very low levels of TSNAs (Foulds et al 2003, Rodu & Godshall 2006). Modern moist snuff products are sold in pre-portioned pouches similar to teabags, but much smaller. Because these products remain stationary in the mouth and generate very little juice, they can be used more discreetly. There is even a recent trend among manufacturers to offer a wide range of non-tobacco flavours (Foulds et al 2003, Rodu & Godshall 2006).

1.5.3 Health implications of tobacco consumption

The UK Medical Research Council (MRC) supported much of the early research into the hazards of smoking and in 1957 was the first national institution in the world to formally accept the evidence that cigarette smoking is a major cause of death (Peto 1994, Doll et al 2004). Several studies in the late 50s, comparing the life histories of patients with lung cancer with those of people without the

disease, showed that the only major difference was that almost all those with lung cancer had been smokers (Peto 1994). Major prospective studies of smoking and death were established independently in Britain and the US, showing strong associations between smoking and death not only from various cancers, but also from respiratory disease, particularly chronic bronchitis, and vascular disease, especially heart attacks (Peto 1994, Doll et al 2004). In 1962 the Royal College of Physicians published the first official report specifying the dangers of smoking. This was followed two years later by the US Surgeon General's report on tobacco. Tobacco dependence is now recognised as a disease by the World Health Organisation (Foulds et al 2004).

Cigarette smoking remains the single largest cause of preventable death and disease. Worldwide there are an estimated 1.2 billion smokers, of whom half will lose ~8 years of life if they are unable to quit. According to projections by the World Bank, 180 million premature deaths caused by tobacco could be avoided during the next 50 years by halving the prevalence of cigarette smoking.

Although smoking prevalence continues to fall slowly towards 20% in some Western countries, tobacco use remains high and rising in many developing countries and is >50% among men in many Asian countries, such as Japan, China and Vietnam (Foulds et al 2004).

In the UK, one in four of the adult population smoke, with much higher levels in deprived sections of society (Moxham 2000). Cessation can greatly improve health prospects, not only from the smoker's perspective, but also from the perspective of the smoker's family and society as a whole, as the health consequences of smoking extend to healthy non-smokers through passive or second-hand smoking (Jorenby 2001). Prolonged cigarette smoking from early adult life can triple age specific mortality rates compared to life-long non-smokers, however, quitting smoking even late in life can regain several years of life expectancy (Doll et al 2004). Due to nicotine's addictive properties it is not surprising that although two thirds of smokers want to quit, and each year about 30-50% try to do so, only 2-5% succeed in remaining abstinent long-term (Moxham 2000, Foulds et al 2004).

1.6 Smoking cessation and harm reduction

The prevention of smoking-related disease requires more than just smoking cessation services. Measures, which might contribute to this goal, include reduction/banning of advertising and promotion, mass media educational and motivational campaigns, restrictions on smoking in public places and at work, and increased taxation of tobacco products. Although it might be desirable to aim for complete exclusion or prohibition of nicotine use, it may be necessary to accept the place of nicotine in society but to use regulation to make nicotine products safer (Britton et al 2001).

In countries with comprehensive tobacco health education programmes and declining smoking prevalence (e.g. Australia, Canada, US & UK), the use of pharmacotherapy for smoking cessation has increased over time and is likely to rise even further as the anti-smoking climate continues to intensify and higher tobacco taxes and further legislation requiring smoke-free public places come into force (Foulds et al 2004). In 1999, the UK government initiated smoking cessation clinics within the National Health Service, providing a comprehensive treatment strategy for nicotine addiction, including the provision of bupropion and all forms of NRT (see section 1.6.1) on prescription and the introduction of specialist smoking cessation services to provide behavioural support to people wanting to stop smoking (Coleman & West 2001, Hajek et al 2007). In 2002, NRT was made available free of charge to most users in an attempt to encourage more smokers to attempt to break the grip of tobacco dependence (Hajek et al 2007).

The main diagnostic criteria for tobacco dependence are: unsuccessful attempts to stop smoking, difficulty controlling tobacco use and previous experience of withdrawal symptoms during a period of abstinence. A major part of the difficulty in quitting is the experience of intense craving for tobacco, combined with a nicotine withdrawal syndrome, which may include symptoms such as increased irritability, restlessness, depression, anxiety, insomnia, poor concentration and hunger. The extremely unpleasant nature of tobacco withdrawal helps explain why many people attempting to stop smoking relapse, often within a matter of hours or days, and many smokers have to make multiple quit attempts before they eventually succeed in remaining abstinent for good (Jorenby 2001, Hughes 2003, Foulds et al 2004).

Another reason why many quit attempts, especially in women, are unsuccessful, is that nicotine is an anorexic drug that gives a sense of satiety, suppressing appetite and increasing energy expenditure, which means that smokers tend to gain weight when they stop smoking (on average ~4 kg).

Unfortunately, dieting appears to increase the risk of relapse. However, some studies suggest that post-cessation exercise not only prevents weight gain but also aids cessation, and both NRT and bupropion have been shown to prevent weight gain. Therefore one option is to encourage exercise and to use a medication initially, postponing any diets (if required) until abstinence is well established and medication levels have been reduced (Hughes 2003).

Most of the pharmacological treatments developed so far are aimed at relieving craving and withdrawal symptoms, and have primarily been licensed as aids to smoking cessation, which restricts their use to a discrete time period (~8-12 weeks) after ceasing tobacco use. However, increasing attention is being given to broadening the indications to include use as an aid to reduced smoking, for long-term nicotine maintenance, or as a treatment for nicotine withdrawal during temporary abstinence (Foulds et al 2004).

Tobacco harm reduction is a relatively novel approach, aimed at minimising tobacco-related risks by substituting smoked tobacco with safer sources of nicotine for those smokers who are unable or unwilling to achieve total nicotine and tobacco abstinence. Switching to low tar/low nicotine cigarettes does not appear to have the desired effect, as smokers tend to compensate to maintain nicotine concentrations by inhaling more deeply and frequently. However, reduction of exposure to cigarettes and associated toxins (carbon monoxide, tar, carcinogens) can be achieved by reducing the amount smoked, switching to smokeless tobacco, or using NRT as a substitute for some or all of the nicotine obtained from smoking. This kind of approach is likely to benefit heavily addicted smokers, as these are often resistant to conventional cessation strategies emphasising complete tobacco and/or nicotine abstinence (Schneider et al 2001, Hughes 2003, Rodu & Godshall 2006).

Successful reduction of the quantity of cigarettes smoked appears to have a beneficial effect on future cessation likelihood, even after controlling for initial smoking level and other variables known to impact on smoking cessation (Falba et al 2004), and there is evidence to suggest that smokeless tobacco products

are generally safer than smoking as they are associated with a lower incidence of cancers and other diseases, probably due to reduced exposure to toxins compared to cigarettes (Rodu & Godshall 2006). Substituting smokeless tobacco products or NRT for some cigarettes may reduce daily smoking without compensation through altered smoking patterns and with reduced "harm" to the individual from carcinogens; however, a relapse to full smoking is likely if substitution is stopped. The nicotine inhalator may be especially suited to a harm reduction approach because it replaces some of the oral and ritual reinforcement aspects associated with smoking, as well as delivering the required nicotine (Schneider et al 2001). Although some long-term reduction and increased interest in quitting have been demonstrated in studies using NRT as a substitute for smoking, doubts remain as to whether patients would persist with such treatment in the longer term, and also whether the smoking reductions achieved are large enough to result in significant improvements in terms of health outcomes (Foulds et al 2004).

There are fears that a harm reduction approach may interfere with the goal of smoking cessation by providing smokers with an "excuse" to continue smoking, believing they can escape health consequences with "harm reduction". At present it is also unclear exactly what constitutes a meaningful reduction in harm. Generally speaking, though, indefinite use of NRT or smokeless tobacco products is considered a preferable alternative to any smoking, while "harm reduction" may be viewed as a "stage of cessation" on the long road to a tobacco-free existence (Schneider et al 2001).

1.6.1 Pharmacotherapy options

1.6.1.1 Nicotine replacement therapy (NRT)

The most commonly used medication for smoking cessation is nicotine replacement therapy (NRT), which is available in a variety of forms. These products deliver nicotine to substitute for that previously derived from tobacco, without exposing the user to the carbon monoxide, tar, and carcinogens found in cigarette smoke (LeSage et al 2006, Hatsukami et al 2005, Fiore et al 1994, Fiore 2000, Ferry et al 2003, Hurt et al 1997, Jorenby 2001). NRT medications are generally well tolerated and most side effects are due to the irritant effect of nicotine, for example skin irritation from using patches (Coleman & West 2001).

They primarily improve cessation efforts by reducing the severity of withdrawal symptoms and cravings, through the provision of an alternative source of nicotine. Most trials have compared one form of NRT with a placebo group or other cessation medication. However, in a trial comparing various NRT products, Hajek and colleagues did not observe any significant differences in terms of efficacy, with continuous validated abstinence rates (at 12 weeks) of 20%, 21%, 24% and 24% for gum, patch, spray and inhalator groups, respectively (Hajek et al 1999).

Given that the central problem is nicotine addiction, nicotine replacement therapy (NRT) is a rational and indeed effective therapy, and has been shown to double quit rates. This is significant in public health terms because of the large number of smokers and relative cost-effectiveness (Moxham 2000, Foulds et al 2004). Although current FDA-approved labelling advises against combining NRT products, using various forms of NRT simultaneously does appear to increase quit rates without increasing side effects (Hughes 2003).

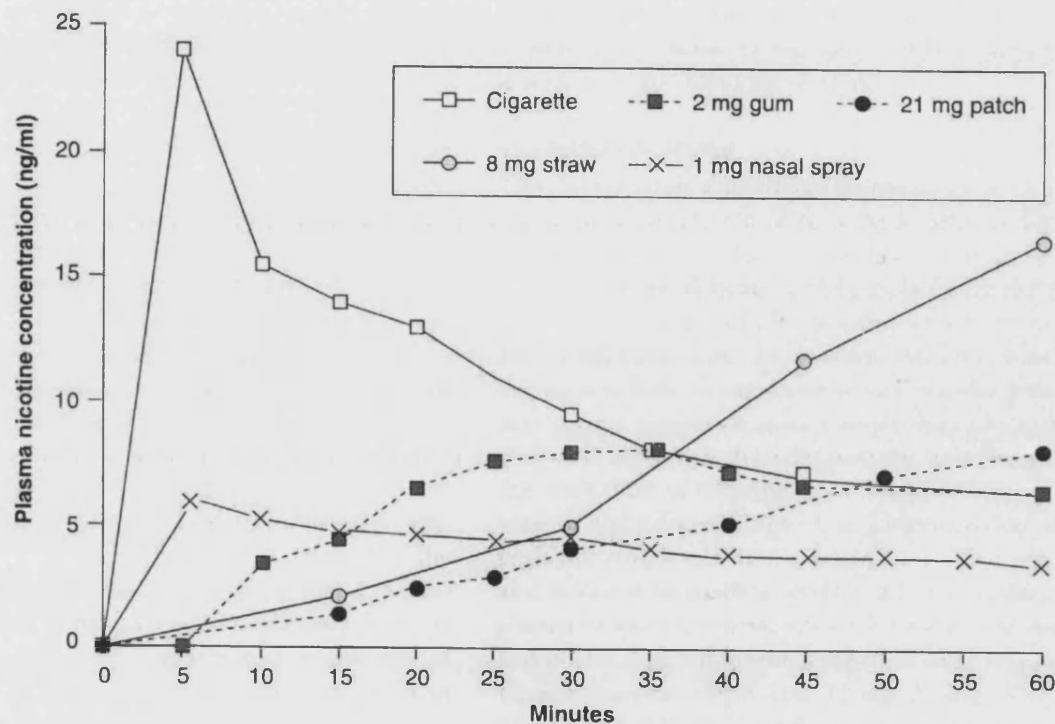
The need for pharmacotherapy for the treatment of nicotine withdrawal during temporary abstinence is likely to increase as more public places become smoke-free. Some smokers, for whom nicotine withdrawal symptoms may present a significant clinical or performance risk, could be required to work in such an environment for extended periods of time. While some NRT products have been licensed for this indication in countries such as Denmark, most have not been evaluated for withdrawal relief over relatively short periods of abstinence (Foulds et al 2004).

The recommended duration of NRT use is currently up to 3 months, but some patients continue to use the medication for longer. Extended use over a longer period of time may be pivotal to a successful quit attempt by more dependent smokers who are likely to be affected by protracted withdrawal discomfort and may therefore require more prolonged access to withdrawal relief medication (Hajek et al 2007).

NRT has been designed to alter reinforcement by changing the pharmacokinetic parameters of nicotine intake (see Figure 1.9). Inhalation of nicotine in smoke is considered to be the most dependence-producing form of administration because of the rapidly established concentrations of nicotine in brain and blood, which is a known factor in the abuse liability of drugs. Although there may be

advantages to rapid nicotine delivery in terms of immediate craving relief, this tends to be correlated with the severity of minor side effects, which in turn adversely affect compliance. Reducing the speed and extent of drug delivery has been central to the development of nicotine treatment for tobacco dependence (Schneider et al 2001, Hughes 2003, Foulds et al 2004).

Figure 1.9: Comparison of venous blood concentrations of nicotine over time for various nicotine delivery systems



Plasma nicotine (ng/ml) increases sharply within minutes of smoking a cigarette and then gradually decreases, whereas NRT gum, patch and straw result in a much more gradual increase. NRT nasal spray achieves a maximal plasma nicotine concentration within minutes, however this maximum is only ~1/5 of that achieved by cigarette smoking. (from Foulds et al 2004)

Transdermal nicotine is available as a 24-hour (7, 14, and 21 mg) or 16-hour patch (15 mg). Absorption from patches is very slow, with plasma nicotine concentration rising gradually over 6-10 hrs, and levels obtained are also lower than after cigarette smoking. The major advantage of the patch is that it requires only a once per day dosage and is more socially acceptable and discreet than nicotine gum. The major disadvantage is that it cannot be used to

alleviate sudden cravings due to the slow rate of absorption (Coleman & West 2001, Hughes 2003, Tutka et al 2005).

Nicotine gum and lozenges are available in 2 and 4 mg doses (for <25 and >25 cigarettes/day smokers, respectively), and the provision of mint and citrus flavoured gum has significantly improved its taste. Absorption from both products is gradual and plasma nicotine concentrations obtained are lower than those found after cigarette smoking. Frequent dosing, and correct chewing technique in the case of the gum, is required in order to achieve adequate plasma nicotine levels. Side effects include jaw ache, nausea, and stomach ache (Hughes 2003, Foulds et al 2004, Tutka et al 2005).

A biphasic buccal adhesive nicotine tablet is being developed, which is designed to provide a steady supply of nicotine similar to the nicotine patch and also a slightly faster delivery of nicotine through the buccal membrane to help relieve initial cravings. In addition, it is intended to provide reasonable patient compliance by being easy to use, as it is applied to the upper gum and no further chewing (like with gum) or puffing (as with the inhalator) is required (Foulds et al 2004).

Nicotine nasal spray is available on prescription as a single dose. The major advantage of the spray is that it provides higher and more rapid nicotine absorption compared to other NRTs, and peak arterial plasma levels are reached within ~5 min after administration. However, this is still less than one tenth the arterial nicotine levels seen with cigarettes. Its major disadvantage is that nasal irritation, lacrimation, rhinitis, coughing, sneezing, and facial flushing are experienced by more than 75% of users (Hughes 2003, Tutka et al 2005).

The nicotine inhalator consists of a plug impregnated with nicotine in a plastic rod. When warm air is pulled through the rod, nicotine is inhaled and absorbed. The inhalator is available as a prescription item in a single dose. Although termed an inhalator, it actually delivers nicotine through the mouth, like gum, not via the lungs. The major advantage of the inhalator is that it replicates the habit feature of smoking, allowing smokers to also wean themselves off the secondary reinforcers of smoking (e.g. handling and oral manipulation of the

cigarette). Its major disadvantage is the need for multiple puffs to obtain sufficient nicotine, and the most common side effect is throat irritation (Schneider et al 2001, Hughes 2003).

A true pulmonary delivery nicotine inhalator, which could deliver nicotine particles to the lungs, allowing it to enter the brain via the arterial system in a manner more like a cigarette, is under discussion. This could reduce acute cravings and increase cessation efficacy, and potentially replace smoking. However, it is difficult to formulate nicotine particles small enough to pass into the lungs in a vapour form, which are still tolerable to the senses. Additionally, the higher dependence potential may create difficulties in obtaining approval for regular use from the regulatory bodies (Foulds et al 2004).

The nicotine "Straw" is a single-use drinking straw containing a loose bead formulation of nicotine bitartrate. It has a fluid permeable plug at the bottom, facilitating the ingestion of the nicotine with the first sip of a beverage. The Straw aims to be more user-friendly than other forms of NRT, and the hand-to-mouth motion may also appeal to smokers as it replicates the habit feature of cigarette smoking to a certain extent. It appears to be well tolerated, though nausea and light-headedness have been reported as side effects. However, speed of delivery does not seem to be improved over other forms of NRT (apart from the patch), and the straw may also have a higher abuse potential. Perhaps the main problem with this product is the risk associated with a potential overdose from a single large dose accidentally ingested by a child or non-smoker with little tolerance to nicotine (Foulds et al 2004).

Another oral delivery method, which has been tested, is nicotine solution drops. Volunteer smokers were provided with the nicotine solution drops to add to beverages of their choice to control urges to smoke. Among the beverages used to dilute the drops were chocolate milk, water, alcoholic beverages and soda. The drops were well tolerated, and the solution provided levels of nicotine, which could assist with smoking cessation. The nicotine solution has the advantage of varied dosing, which can be more finely adjusted to treat different intensities of dependence. The slow absorption rate may reduce the

dependence potential, but also reduces the solution's utility as an acute craving rescue medication (Foulds et al 2004).

The rate of nicotine metabolism by an individual (see section 1.2) can predict cigarette consumption and smoking persistence, but it can also impact on the effectiveness of NRT as a treatment for smoking cessation. Smokers with higher nicotine metabolite ratios tend to have more severe cravings to smoke during the first week of abstinence, which could increase relapse liability. Such smokers might therefore best be advised to try a form of NRT other than patches (where the nicotine dose is fixed), so that they can titrate their nicotine intake according to the severity of cravings, or they could be prescribed a non-nicotine medication (see below) (Lerman et al 2006). Smokers who have previously failed to remain abstinent after a quit attempt with one type of NRT could be encouraged to try again using another product, as some patients appear to respond better to the steady-state nicotine delivery from patches, whereas others have an increased chance of success with more acute dosing forms. And finally, smokers who have tried several NRT products unsuccessfully might benefit from using bupropion (see below), or a combination of the two (Shiffman et al 2004a).

1.6.1.2 Non-nicotine smoking cessation medications

In addition to NRT, the potential of a variety of agents as smoking cessation medications has been explored. Pharmacotherapies that do not contain nicotine can be roughly divided into several groups: drugs targeting nicotinic receptors or enzymes involved in the metabolism of nicotine, compounds acting on dopaminergic and/or noradrenergic systems, and a variety of other agents that do not fit in either of the above categories.

1.6.1.2.1 Drugs targeting nAChRs or nicotine metabolism

nAChR antagonism

Nicotine acts at a variety of nAChR subtypes in the CNS to produce its reinforcing effects. Blocking these receptors with an antagonist should reduce the reinforcing effects of cigarette smoking and promote abstinence.

Mecamylamine, a non-specific nicotinic antagonist, attenuates nicotine-evoked DA release from rat striatum (Puttfarcken et al 2000), effectively blocking the rewarding effects of nicotine, and has been shown to decrease nicotine self-administration and attenuate cue-induced reinstatement of self-administration behaviour in rats (Liu et al 2007). The drug, which was originally used as an antihypertensive, has also been shown to enhance short-term smoking cessation (Rose et al 1998), especially in conjunction with transdermal nicotine patches. It does not appear to precipitate withdrawal in smokers (Eissenberg et al 1996, Rose et al 2001), unlike in rats, which displayed signs of nicotine withdrawal after mecamylamine administration (Malin et al 1992, O'Dell et al 2007). At the lower doses used for smoking cessation, patients also do not experience the severity of side effects, which made the drug unpopular for the treatment of hypertension (Foulds et al 2004, Siu & Tyndale 2007).

nAChR partial agonists

Partial agonists at nAChRs are also being explored as potential smoking cessation medications. Such compounds are hypothesised to reduce nicotine-mediated activation of the dopaminergic system via partial blockade of nAChRs, thus lowering the rewarding effects of nicotine. In addition, by acting as weak agonists, they could also stimulate dopamine release in the mesolimbic region and reduce craving and withdrawal during abstinence (Foulds et al 2004).

Cytisine (from *Cytisus laburnum* L.) is a partial agonist at $\alpha_4\beta_2$ nAChRs (Abin-Carriquiry et al 2006), and has been used as a smoking cessation aid in Bulgaria since the 1960s (Tutka & Zatonski 2005). The drug is widely used throughout central and eastern Europe and appears to be well tolerated without many side effects. Cytisine has a mechanism of action similar to nicotine but with low toxicity and short-lived effects. In contrast to nicotine, it undergoes minimal metabolism and 90-95% appears to be excreted unchanged. Cytisine has been observed to reduce craving and withdrawal signs, and abstinence rates are similar to those achieved with NRT, although the majority of studies were performed in the 1960s and 1970s and showed several design and analysis shortcomings, such as a lack of controls, limited follow-up periods, or inadequate verification of abstinence criteria (Foulds et al 2004, Tutka & Zatonski 2005, Siu & Tyndale 2007).

Varenicline (Chantix (US), Champix; Pfizer pharmaceuticals) is structurally similar to cytosine and is also a partial agonist at $\alpha_4\beta_2$ nAChRs (Foulds et al 2004, Coe et al 2005, Rollema et al 2006, Siu & Tyndale 2007, Smith et al 2007). In Phase II clinical trials, continuous quit rates were higher with varenicline than with bupropion or placebo. Phase III studies have shown mixed results in terms of continuous abstinence levels; however they did demonstrate that varenicline provided significantly greater benefit compared with placebo, when examining the subjective effects associated with smoking. Overall, the adverse events observed were relatively mild, most commonly nausea, and the percentage of patients who discontinued the studies due to adverse events was comparable to bupropion and placebo (Siu & Tyndale 2007).

Varenicline was fast-track approved as a monotherapy by the Food and Drug Administration (US) in May 2006. Clinically significant drug-drug interactions have not been observed, and in vitro studies did not find any effects on cytochrome P450 enzyme activity (Rollema et al 2006). The safety of co-administration of varenicline with NRT products has not been well established, although it is thought that the incidence of adverse events (nausea, headache, vomiting, dizziness etc) may be increased with combination therapy (Zierler-Brown et al 2006).

Inhibitors of CYP2A6 activity

In terms of smoking behaviour, slower metabolisers ($\leq 50\%$ nicotine metabolism activity) consume fewer cigarettes per day and are at lower risk of becoming dependent smokers. They also smoke for shorter durations and are more likely to quit compared with normal metabolisers. Reducing nicotine metabolism through CYP2A6 inhibitors such as methoxsalen and tranylcypromine appears to increase plasma nicotine levels in abstinent smokers using NRT, as well as decreasing the desire to smoke. Use of CYP2A6 inhibitors alone might also reduce smoking and increase quitting, and Phase II clinical trials are being conducted using this approach (Siu & Tyndale 2007).

NNK, one of the most potent procarcinogens in tobacco smoke, is activated by CYP2A6. Epidemiology studies have found that poor metabolisers have a significantly lower risk for developing lung cancer, even after adjusting for the number of cigarettes smoked. In animal models, administration of methoxsalen

reduced the formation of NNK-induced lung tumours, and in human smokers it appears to inhibit the activation of NNK. Therefore, in addition to enhancing smoking cessation, CYP2A6 inhibitors might also provide some harm reduction in smokers attempting to quit (Siu & Tyndale 2007).

1.6.1.2.2 Drugs acting on dopaminergic and/or noradrenergic systems

Stimulation of presynaptic nAChRs increases the release of dopamine (DA) and noradrenalin (NA), mediating the rewarding effects of nicotine. During abstinence, the lack on nAChR stimulation results in reduced neurotransmitter release, with abstinent smokers experiencing a variety of unpleasant withdrawal symptoms as a result. Cessation medications targeting dopaminergic and/or noradrenergic systems aim to restore this balance by increasing extracellular neurotransmitter levels, either by stimulating DA / NA receptors directly, or by reducing DA / NA uptake via their respective transporters. The most important compound in this category, and the only one currently licensed for first-line treatment of nicotine addiction, is bupropion.

Bupropion was the first non-nicotine medication to demonstrate efficacy in the treatment of tobacco dependence and was originally marketed as an atypical antidepressant (Wellbutrin®; GlaxoSmithKline) in the US (approved by the FDA in 1984). Since 1997 it has been used for smoking cessation under the trade name Zyban (Slemmer et al 2000, Foulds et al 2004, Siu & Tyndale 2007) and appears to work equally well in smokers with or without a history of depression (Hayford et al 1999, Hughes 2003).

Bupropion reduces DA and NA uptake through inhibition of their respective transporters, thus mimicking the action of nicotine by elevating extracellular catecholamine levels (LeSage et al 2006, Hatsukami et al 2005, Fiore et al 1994, Fiore 2000, Ferry et al 2003, Hurt et al 1997). At high concentrations it also inhibits the firing of locus coeruleus noradrenergic neurons projecting to the hippocampus, a region implicated in drug dependence (Cooper et al 1994, Slemmer et al 2000, Siu & Tyndale 2007). In addition, bupropion can act as an antagonist at nAChRs and appears to block nicotine-induced antinociception, motor effects, hypothermia, and convulsive effects in mice (Slemmer et al 2000), as well as reducing activation of $\alpha_3\beta_2$, $\alpha_4\beta_2$, and α_7 nAChRs (in oocytes) by nicotine with some degree of selectivity (~50 and 12 times more effective in

blocking $\alpha_3\beta_2$ and $\alpha_4\beta_2$ than α_7 , respectively). On the other hand, bupropion failed to reduce nicotine self-administration, attenuate nicotine discrimination or modify aversive effects of nicotine in rats (Shoaib et al 2003), possibly due to its dopamine uptake blocking effects. However, it has been shown to attenuate somatic signs of nicotine withdrawal in rats (Lake et al 2001), suggesting that it may be effective because it attenuates the increases in depression, difficulty concentrating, irritability etc. associated with abstinence from nicotine (Shiffman et al 2000).

Bupropion roughly doubles long-term abstinence rates compared with placebo or no medication, similar to NRT. Sustained one-year abstinence rates average ~20%, and when combined with NRT quit rates further increase slightly (Coleman & West 2001, Hughes 2003). Although bupropion has a relatively good side-effect profile for an antidepressant, a significant proportion of patients find its mild stimulant effects (jitteriness, insomnia) and other side effects (headache, dry mouth) unpleasant. Bupropion also carries a small increased risk of seizures, although at doses used for smoking cessation, this only affects about 1 in 1000 users (Coleman & West 2001, Hughes 2003, Foulds et al 2004, Siu & Tyndale 2007).

Bupropion metabolites are also pharmacologically active (Damaj et al 2004). CYP2B6, the main enzyme metabolising bupropion to hydroxybupropion, is subject to genetic variability (see section 1.2), which can alter enzyme activity and appears to be associated with an increase in craving and higher smoking relapse rates. However, alleles decreasing the metabolism of bupropion could lead to increased plasma levels of the drug, potentially resulting in a larger therapeutic impact (Lee et al 2007).

Several other compounds that act via dopaminergic and/or noradrenergic mechanisms are also being investigated as potential smoking cessation medications:

| Compound | Likely mechanism | Characteristics |
|---------------|------------------------------------|---|
| Reboxetine | - selective NA uptake inhibitor | - dose-dependently decreases nicotine self-administration |
| Nortriptyline | - NA (& 5-HT) reuptake blocker | - reduces withdrawal - cessation rates comparable to bupropion - poor side-effect profile |
| Clonidine | - α_2 -adrenoceptor agonist | - reduces withdrawal - similar efficacy to bupropion - poor side-effect profile |

1.6.1.2.3 Other therapeutic approaches

Finally, several other compounds have been proposed as possible candidates for the treatment of nicotine addiction; however, there is only limited safety and efficacy data available for the majority of these drugs.

| Compound | Likely mechanism | Characteristics |
|------------------|---|--|
| Selegiline | - irreversible MAO-B inhibitor | - reduces craving in combination with NRT patch - possible concern over irreversible effects |
| Naltrexone | - opioid receptor antagonist | - potential utility with concurrent alcohol problem - side effects (mainly sedation) & lack of efficacy |
| Rimonabant | - selective cannabinoid (CB1) receptor antagonist | - variable efficacy in trials - reduced weight gain - well-tolerated |
| Glucose/Dextrose | - reduces carbohydrate craving | - variable efficacy in trials - good safety - low cost |

1.6.2 Psychosocial treatment programmes

Behavioural therapy focuses on a problem-solving approach, where smokers are encouraged to think about when they are likely to smoke and plan something to distract themselves when the urge strikes. This approach can increase quit rates by a factor of 1.5 – 2.1. Social support, in the form of encouragement, caring, and concern, can increase quit rates by a factor of 1.3 – 1.5. This kind of support can come from both healthcare providers and from family, friends, and other community members (Jorenby 2001, Hughes 2003, Foulds et al 2004).

Behavioural and supportive therapies were developed initially for use in individual or group therapy formats. However, less than 5% of smokers attend such therapy. Written materials do not appear to be effective, however, delivering behavioural therapy via the telephone has been shown to increase quit rates by a factor of 1.2. Although less effective, this format is so much more acceptable that it has a bigger impact than group or individual counselling. Whether therapy could be delivered via the Internet is being investigated (Hughes 2003, Foulds et al 2004).

An important step towards improved tobacco dependence treatment comes from combining existing and new pharmacotherapies with effective behavioural therapy (Foulds et al 2004). There is evidence that counselling interventions can increase the success rates of pharmacological interventions, with part of the effect attributable to better compliance with medication use over a longer time period. Generally, the more intensive the psychosocial component is, the more effective the overall treatment appears to be, and tailoring behavioural support materials specifically to the smokers' needs appears to be more effective at encouraging abstinence than giving general advice (Shiffman et al 2001, Coleman & West 2001). Integrated medication and behavioural therapies have also been adapted for use in subgroups of smokers, such as those with schizophrenia and depression, and more of these tailored behavioural-pharmacological interventions could be used in the future (Foulds et al 2004).

However, even when behavioural therapy is combined with the most effective pharmacological treatments available, long-term tobacco abstinence rates are typically <30%. There is, therefore, a need to develop new treatments or components of treatment, which can be widely used and will increase cessation rates among dependent tobacco users who try to give up smoking (Foulds et al 2004).

One approach has been the development of vaccines to combat addiction. These are discussed in more detail in section 1.8. However, in order to better understand this approach, some basic knowledge of the immune system and the mechanisms behind immunotherapy in general is required.

1.7 The immune system

(The following sections are largely based on Parham P. The immune system. 2000. Garland Publishing/Elsevier Science Ltd.)

The skin is the human body's first defence against infection. It forms a tough impenetrable barrier; however this barrier can be breached by physical damage, such as wounds, burns, or surgical procedures, which exposes soft tissues and renders them vulnerable to infection.

The epithelia lining the respiratory, gastrointestinal, and urogenital tracts are continuous with the skin. On these internal surfaces, the impermeable skin gives way to tissues designed for communication with their environment, making them more vulnerable to microbial invasion. Such mucosal surfaces are continually bathed in the mucus they secrete, containing glycoproteins, proteoglycans and enzymes, to protect the epithelial cells from damage and help to limit infection.

Skin and mucosa usually provide a well-maintained physical and chemical barrier, which prevents most pathogens from gaining access to the cells and tissues of the body; however, when that barrier is breached and pathogens gain entry to the body's soft tissues, the defences of the immune system are brought into play.

1.7.1 Immune responses

The body has two types of response to invasion by a pathogen – the innate immune response and the adaptive immune response. Although the recognition mechanisms of these responses differ, the means used to destroy pathogens after their identification are common to both. Innate immune defences are present in vertebrates and invertebrates, but only vertebrates appear to have evolved adaptive immune responses.

1.7.1.1 Innate immune response

Innate immunity provides an immediate defence against infection, which is activated as soon as a pathogen is detected. These defences are always available and include physical barriers (skin and other epithelia), microbicidal proteins and complement, phagocytic cells (macrophages & neutrophils) and the cytokines they produce, as well as natural killer (NK) cells.

The cells and molecules involved in the innate immune response identify common classes of pathogen by means of recognition molecules. These proteins bind to distinctive biochemical features on the pathogen surface, which distinguish microbes from mammals. Soluble recognition molecules, such as mannose-binding protein (MBP), bind carbohydrates characteristic of bacterial cell surfaces. Once bound to a bacterium, MBP initiates a series of enzymatic reactions involving a group of plasma proteins known as complement. Binding of complement to a pathogen (opsonisation) flags it as something to be destroyed by phagocytes. Complement proteins bound to pathogens engage specific complement receptors on the surface of the phagocyte, triggering the engulfment and destruction of the complement-coated pathogen. Phagocytes also secrete cytokines, chemokines and inflammatory mediators, which are involved in activating other cells, inducing a local state of inflammation in the infected tissue, and recruiting more phagocytes to the site of infection.

Inflammation is central to the innate immune response, and NK cells, which recognise and kill virus-infected cells, are activated by the various cytokines and inflammatory mediators produced by phagocytes.

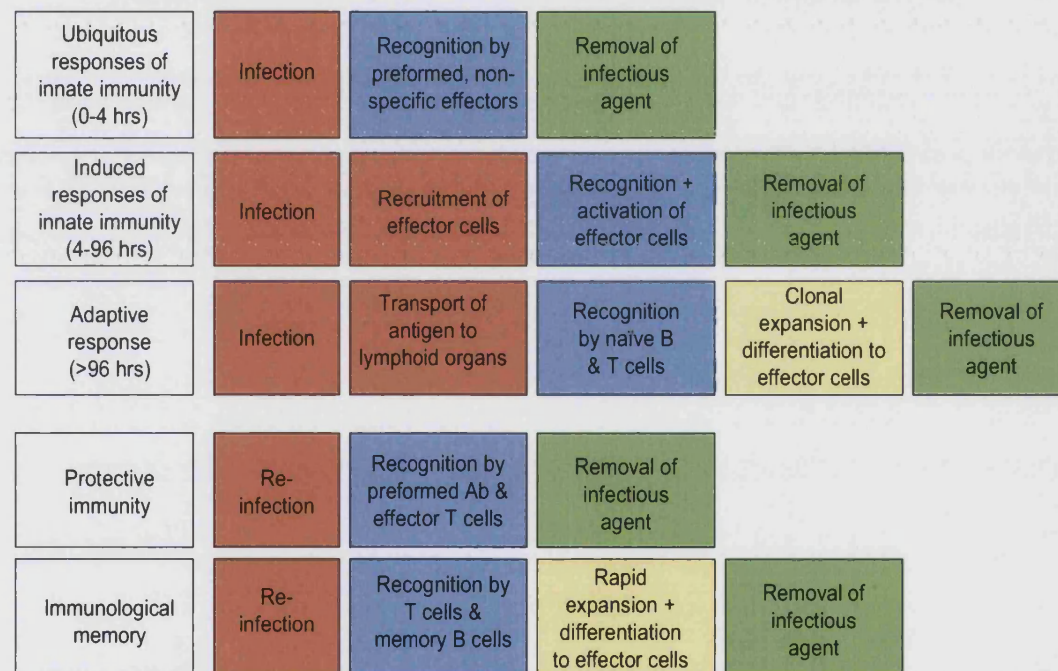
Innate immunity has no specificity for particular antigens and does not produce a specific immunological memory of an infection. The components of the innate

immune response act in concert to clear an infection or to contain it until the adaptive immune response develops.

1.7.1.2 Adaptive immune response

Most infections are highly localised and can be cleared by the innate immune response (see Figure 1.10). An adaptive immune response is only mounted if the infection spreads to the secondary lymphoid tissues, and its initiation depends on the inflammatory cytokines produced by the cells involved in innate immune responses. The main aim of adaptive immunity is to improve pathogen recognition.

Figure 1.10: Characterisation and stages of the immune response



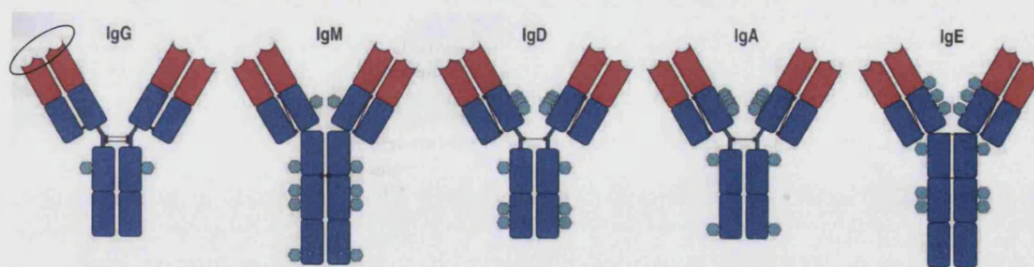
The innate immune response consists of immediate responses (complement activation, macrophage phagocytosis) and responses induced by inflammatory cytokines within a few hours of infection. Effects of the adaptive immune response are seen only from 4 days onwards.

After termination of an infection, effector cells and molecules produced in the primary response continue to provide protective immunity but gradually decline. Immunological memory provides long-term protection, enabling a rapid and much stronger secondary response in the event of re-infection. (adapted from Parham 2000)

Adaptive immune responses are mediated by lymphocytes (B and T cells), which collectively have the ability to recognise a vast array of antigens (see Figure 1.10). An individual lymphocyte expresses receptors of a single and unique antigen-binding specificity, so any given pathogen stimulates only a small subset of lymphocytes, which recognise antigens derived from that particular pathogen. This helps to focus the adaptive immune response on the specific pathogen in question.

B and T cells recognise different forms of antigen: B cell immunoglobulin receptors bind whole molecules and intact pathogens, whereas T cell receptors recognise only short peptide antigens bound to major histocompatibility complex (MHC) molecules on cell surfaces. In the secondary lymphoid tissues, the presentation of pathogen-derived antigens to naive circulating T cells causes the pathogen-specific T cells to divide and differentiate into effector T cells. Effector T_H1 cells and CD8 T cells travel in the lymph and blood to the site of infection and initiate the destruction of extracellular pathogens and infected host cells by macrophages upon recognition of peptides presented by MHC molecules. Effector T_H2 cells remain mainly in the lymphoid tissues, where they help to activate pathogen-specific B cells, which differentiate into plasma cells and produce antibodies.

Figure 1.11: Structure of antibodies and comparison of isotypes



Antibodies consist of two light and two heavy chains, containing constant (blue) and variable regions (red). The site of antigen binding is also indicated (circle).

Heavy chains are longer in IgM and IgE isotypes than in IgG, IgD and IgA, and there are also differences between isotypes in terms of the hinge region and disulfide bonds linking the chains, and in the distribution of carbohydrate groups (turquoise). (adapted from Parham 2000)

Antibodies are Y-shaped molecules consisting of two different polypeptides: two identical heavy chains and two identical light chains (see Figure 1.11). Both types of polypeptide have a constant region, which is identical in amino-acid sequence from one antibody to another, as well as an amino-terminal variable region, which differs in amino acid sequence from one antibody to the next.

These variable regions contain the sites involved in antigen recognition and binding, and the differences in amino acid sequence create a vast variety of binding sites that are specific for different antigens, and thus for different pathogens. The constant regions of antibodies contain binding sites for phagocytes and inflammatory cells, as well as for complement proteins.

Antibodies therefore act as molecular adaptors, bringing together pathogens and effector cells molecules designed to destroy the pathogen.

There are several types of constant region, known as isotypes (IgA, IgD, IgE, IgG, and IgM), differing in their heavy-chain constant regions (see Figure 1.11), which have specialised effector functions:

Cell-surface IgM and IgD form the antigen receptors on circulating B cells, which have yet to encounter antigen. IgM is the first antibody isotype secreted in an immune response, with antibodies of other isotypes emerging as the immune response matures. IgM, IgA and IgG are the main antibodies present in blood, lymph and connective tissues. IgG antibodies facilitate the engulfment and destruction of pathogens and toxins by phagocytes, which bind to the constant regions of the IgG heavy chains with their cell-surface receptors. IgM bound to a pathogen's surface typically activates the complement system, facilitating uptake and phagocytosis of the pathogen. In response to worms and other parasitic infestations, IgE bound to mast cells can trigger a strong inflammatory reaction thought to help expel or destroy the parasite. However, in developed countries, IgE is most often associated with unwanted allergic reactions to innocuous substances.

As well as clearing the infection, a successful primary adaptive immune response also establishes a state of long-term protective immunity. A subsequent encounter with the same pathogen therefore provokes a faster and stronger secondary immune response. This is a result of antibodies and clones of long-lived B and T cells formed during the primary response. In a person possessing protective immunity, the infection is usually cleared before it

produces any symptoms. The purpose of immunisation is to produce such a state of protective immunity against a particular pathogen or, in the case of this thesis, against cotinine.

1.7.2 Immunity

Immunity, or the ability to resist infection, can be achieved by two main approaches: active immunisation (vaccination) or passive immunisation.

1.7.2.1 Active immunity / vaccination

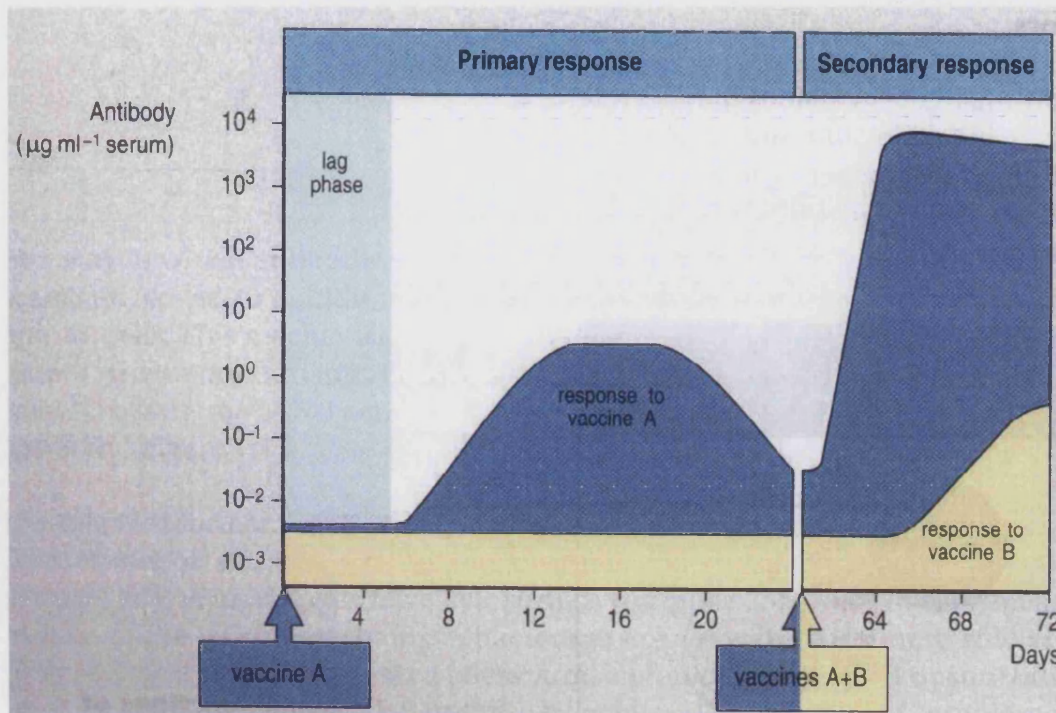
Active immunity is acquired naturally as a result of bacterial and viral infections, but can also be conferred artificially as a result of vaccination. The response of the immune system is identical in both cases. Active immunisation was first used against smallpox, after Edward Jenner showed that inoculation with cowpox virus offered protection against the related smallpox virus. Jenner called his procedure vaccination after vaccinia (lat. *vaccus* = cow), the name given to the mild disease produced by cowpox.

Vaccination involves the deliberate immunisation of healthy people with some form of a pathogen or its component antigens. It induces a protective immunity, which prevents subsequent infection with the same pathogen from causing disease by providing prior exposure to an infectious agent in a form that cannot cause disease. This gives the immune system the opportunity to gain the experience needed to mount a protective response with little risk to health or life. Antigen injected into the host is recognised as foreign by the host's immune system, leading to an immune response and the production of antibodies. After an initial vaccination, the immune system keeps a memory of the antigen and is able to produce large amounts of antibodies relatively quickly upon re-exposure (see Figure 1.12). Booster shots may be used to stimulate and intensify the immune response at regular intervals if required (Foulds et al 2004).

Vaccination can occasionally induce an autoimmune reaction, resulting in the host's immune system mounting an attack against host cells. Vaccination against A β showed encouraging results in preclinical trials, reversing memory deficits in amyloid depositing mice; however, a clinical trial investigating the benefits of anti-A β vaccination in Alzheimer patients had to be cut short due to

several patients developing meningoencephalitic symptoms after vaccination (Morgan 2006).

Figure 1.12: Effects of vaccination - comparison of primary and secondary immune response



After immunisation with vaccine A, antibodies against pathogen A are gradually produced as the immune response develops (blue). The primary response eventually subsides over time.

A second immunisation with vaccine A on day 60 results in an immediate secondary response within days that is orders of magnitude greater than the primary response.

Simultaneous administration of vaccine B produces a typical primary response to pathogen B (yellow), demonstrating the specificity of the secondary response for vaccine A. Responses are measured as pathogen-specific antibodies present in the blood over time. (from Parham 2000)

Many bacterial diseases are a result of the effects of toxic proteins secreted by the bacteria (diphtheria, tetanus). Vaccines against these pathogens are made by purifying the respective toxin – diphtheria toxin or tetanus toxin – and treating it with formalin to destroy its toxic activity. These inactivated proteins, known as toxoids, retain sufficient antigenic activity to provide protection against the disease when used in a vaccine. Less immunogenic molecules can be

covalently coupled to a carrier protein, which provides antigenic peptides required to stimulate an immune response. Such vaccines are known as conjugate vaccines. The vaccine examined in this thesis is such a conjugate vaccine (see section 1.8, 1.9 & Chapter 2), consisting of cotinine molecules, which are normally too small to be recognised by the immune system, coupled to tetanus toxoid, which acts as a highly immunogenic carrier protein to maximise the immune response achieved as a result of immunisation.

1.7.2.2 Passive immunity

An alternative strategy for protection is by passive immunisation. Passive immunity is conferred by injecting specific antibodies to provide protection against a given pathogen or toxin. It differs from active immunity, both in the antibody source and the degree of protection provided. Instead of being made by an individuals' own plasma cells, antibodies are obtained from the serum of an immune human or animal donor. As a result, the patient's B cells are not challenged by antigens, immunological memory does not occur, and the protection provided by the "borrowed" antibodies ends when they naturally degrade in the body. Passive immunity occurs naturally when a mother's antibodies cross the placenta and enter the foetal circulation. For several months after birth, the baby is protected from all the antigens to which the mother has previously been exposed (Marieb 1998). Passive immunity can also be artificially conferred when immune sera are used to treat poisonous snake bites (antivenom), botulism, rabies, and tetanus (antitoxin). These rapidly fatal diseases would kill a person before active immunity could be established, however the donated antibodies provide the immediate protection required, although their effect is relatively short-lived (2-3 weeks).

Immune sera contain a mixture of antibodies of varying specificity and affinity. Monoclonal antibodies arise from a single clone of antibody-producing cells and thus all have identical antigen-binding sites and are of the same isotype. They can be produced on a large scale from hybridoma cell lines, providing large quantities of identical antibodies required for diagnostic and therapeutic applications. Mouse monoclonal antibodies of a desired specificity can be made quite easily but are limited in their therapeutic use because the human immune system recognises them as foreign and mounts an immune response to

eliminate them. By use of genetic engineering techniques, highly specific antigen recognition sites from the variable region of mouse antibodies can be combined with the constant regions of human antibodies, conferring the antigen specificity of the mouse antibody on the human antibody. This results in chimeric or humanised antibodies, depending on the relative contribution of the two species. Humanised antibodies are sometimes used in the treatment of cancer, as they can be raised against epitopes expressed only by the cancerous cells, which allows them to bind to these cells and direct the immune response against them.

1.7.3 Use of adjuvants

The term "adjuvant" is derived from the Latin (adjuvare = to help), and is used to refer to any material that helps to activate and direct the innate and adaptive immune responses, leading to an early, high and long-lasting immune response to an otherwise poorly immunogenic antigen. Adjuvants have been used for more than 80 years to make immunisations more efficient, thus saving on vaccine production costs (Gupta & Siber 1995, Schijns 2003).

Adjuvants form a highly heterogeneous group of compounds, both chemically and with regard to their mechanism of action. They facilitate the delivery of antigen to the secondary lymphoid organs for a sufficient period of time to evoke an immune response, and provide either a non-self, microbial signal, or a host-derived danger signal from stressed cells, both of which are essential for induction of co-stimulatory molecules. Several adjuvants are known to evoke tissue damage and associated cell stress, which may further enhance activation of the immune system. Adjuvant preparations can sometimes contain several substances with different mechanisms of action, such as Freund's adjuvant, which consists of an emulsion of mineral oil and killed mycobacteria, and contains elements of the bacterial cell-wall skeleton and other antigenic peptides. However, this potent adjuvant is only used in preclinical studies, due to its side-effect profile (Gupta & Siber 1995, Schijns 2003).

A number of problems are encountered in the development and use of adjuvants for human vaccines, the biggest of which, particularly in routine childhood vaccines, is the toxicity and adverse side effects of most adjuvant formulations. The choice of adjuvants for human vaccination tends to reflect a

compromise between the need for efficacy and an acceptably low level of side effects. The most common adjuvants used in humans are aluminium hydroxide (alum) and aluminium phosphate, although calcium phosphate and oil emulsions are also used occasionally. Much progress has been made on development, isolation and chemical synthesis of alternative adjuvants such as derivatives of muramyl dipeptide, monophosphoryl lipid A, liposomes, QS21, MF-59 and immunostimulating complexes (ISCOMS) (Gupta & Siber 1995), and GSK have developed several proprietary adjuvants for possible application in humans that are used in the experiments described in this thesis.

1.7.4 Effects of nicotine on the immune system

Nicotine is thought to contribute to the immunosuppressive effects of chronic smoking by inducing a state of anergy in lymphocytes, resulting in impaired responses of lymphocytes to antigen-induced signalling (Geng et al 1995 & 1996).

In animal studies, nicotine, obtained by self-administration or from nicotine patches, as well as chronic exposure to cigarette smoke, has been shown to suppress antibody- and cell-mediated immune responses, as well as decreasing inflammatory responses and expression of proinflammatory cytokines (Kalra et al 2000, 2002, 2004). Treatment of rats with nicotine, but not cotinine, for 3-4 weeks inhibited both T cell-dependent and -independent immune responses (Geng et al 1995). Adolescent (postnatal (PN) day 30-37) chronic nicotine exposure elicited lasting deficiencies in T cell mitogenesis in rats, which were dose-dependent (nearly 50% reduction at the high dose). These effects disappeared initially after termination of nicotine treatment, but re-emerged in young adulthood (PN65). Administration of the same total dose of nicotine as twice-daily injections did not result in such deficits in T cell function (Navarro et al 2003). In mice, $\alpha 7$ and $\alpha 4\beta 2$ nAChR subtypes appear to be expressed on B lymphocytes and/or macrophages, and deficiency of these subunits has been observed to affect immune responses, with macrophages from $\alpha 7$ -deficient mice producing elevated levels of inflammatory cytokines (Wang et al 2003, Skok et al 2005).

Tobacco smoking appears to suppress the immune system in humans, and several studies have shown that tobacco smokers have lower levels of IgG and IgA, and higher levels of IgE than non-smokers, which may contribute to the propensity for respiratory infections observed in smokers. IgG2 levels are most affected with a reduction of mean levels by ~40% compared with non-smokers, which may help to explain why smoking is a risk factor for invasive pneumococcal infections, since antibodies produced in response to infection by such bacteria belong predominantly to the IgG2 subclass (Gyllen et al 2004, Kalra et al 2004). Interestingly, smokers have a lower incidence of some diseases (ulcerative colitis, endometriosis and Parkinson's disease among others), many of which are inflammatory in nature or have an inflammatory component (Kalra et al 2004).

The mechanisms by which smoking affects serum antibody levels is unclear, although there is evidence for a dose-response relationship (lower levels of IgG with increased smoking) and the effect appears to be reversible after smoking cessation. The cigarette smoke component responsible for this effect has not yet been identified, but animal and in vitro studies have implicated nicotine as a major factor (see above). Both nicotine and cotinine (at 100-fold higher concentrations than nicotine) have been shown to modulate immunoglobulin synthesis by human lymphocytes in vitro (Fischer & König 1994). However, smokeless tobacco or nicotine replacement therapy does not appear to significantly affect immunoglobulin levels in vivo (Gyllen et al 2004).

1.8 Development of vaccines to combat addiction

Drugs of abuse are a major public health problem, with millions of users worldwide taking some form of addictive substance (cocaine, heroin, nicotine etc). Pharmacotherapy approaches for the treatment of drug abuse do exist, however most have only a very low success rate, with the vast majority of patients undergoing treatment relapsing into drug taking (Anton & Leff 2006, LeSage et al 2006).

Drugs of abuse exert their reinforcing effects by binding to receptors in the CNS, causing neurotransmitter release and activation of the mesolimbic reward pathway (see section 1.4). Repeated stimulation of the reward pathway leads to

neurobiological adaptations and the development of dependence, with drug users experiencing withdrawal symptoms upon abstinence from the drug. The receptors to which these drugs bind are found throughout the brain, which means that pharmacological treatments, such as receptor agonists or antagonists acting at these receptors, almost invariably produce undesirable side effects (Nutt & Lingford-Hughes 2004, Anton & Leff 2006).

Blocking the actions of an addictive drug at its receptor should prevent or at least reduce reinforcement of drug-taking behaviour. However, such antagonists can also prevent/reduce the binding of endogenous substances at their receptors (e.g. naltrexone & naloxone block the binding of enkephalins & endorphins at opioid receptors), causing negative emotional effects in patients (Anton & Leff 2006). The use of agonists mimicking the actions of a drug of abuse should help to alleviate withdrawal symptoms experienced during abstinence, however they can lead to the development of tolerance to the treatment agent and withdrawal from it when the treatment is terminated, such as with methadone treatment for heroin addiction (Anton & Leff 2006).

As current treatment options for drug abuse are somewhat inadequate, a new avenue is being explored – immunotherapy. Immunotherapies for drug abuse are being developed primarily as therapeutic vaccines, designed for treating patients who already have a disease (i.e. addiction), as opposed to a prophylactic vaccine used to prevent the development of addiction before an individual has ever been exposed to the drug (Kosten et al 2005).

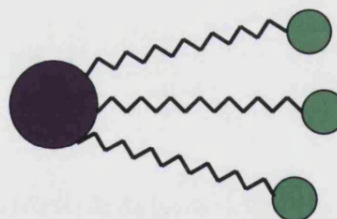
Drugs of abuse are small molecules (molecular weight <1000 Da), which readily cross the blood-brain barrier, while larger molecules such as antibodies (molecular weight ~150 kDa) normally cannot. Thus, any drug bound with high affinity to an antibody cannot cross the blood-brain barrier and therefore cannot enter the brain (Kosten et al 2005). After vaccination, antibodies bind a significant proportion of the drug dose and prevent it from entering the brain. This leads to a reduction in the effects of the drug, even at doses exceeding the apparent binding capacity of the antibodies. Provided the reduction of drug effect is sufficiently large, it could lead to a decrease in drug use or help to reverse a drug overdose (Kosten et al 2005).

Targeting the drug molecules rather than the receptors they bind to should reduce the likelihood of side effects on normal neurotransmission, especially as the antibodies remain primarily in the circulatory system. Preventing the drug from binding to its target receptors could also help to weaken the cycle of drug taking by reducing the psychostimulant effects and abolishing the drug's reinforcing actions. However, the specificity of the antibodies raised by vaccination against a given drug also means that they are ineffective if an addict switches to another drug of abuse (Carrera et al 1995, Nutt & Lingford-Hughes 2004, Kosten et al 2005).

As a general rule, immune responses are not generated against molecules of less than ~10 kDa, which is substantially larger than any drug of abuse. In order to make a vaccine against such a small molecule, the drug must be covalently linked to a very immunogenic protein (see Figure 1.13), which is usually done by synthesising a derivative of the drug (hapten), containing a linker arm with a reactive chemical species. Frequently, proteins derived from bacterial toxins are used in the production of these conjugate vaccines, as they elicit a strong immune response (Foulds et al 2004). The precise molecular orientation and spacing of these drug haptens on the protein surface are critical factors for an optimal immune response (Kosten et al 2005).

Figure 1.13: General structure of conjugate vaccine

Several hapten molecules (turquoise) are attached to a highly immunogenic carrier protein (purple) by means of linker arms (only 3 shown for clarity).



Immunotherapy has two immediate clinical applications in drug abuse treatment: to combat drug overdose and to reduce relapse to drug use in addicted patients. The specificity of antibodies, their lack of addiction liability, minimal side effects and long-lasting protection against drug use offer major benefits over conventional small molecule agonists and antagonists. It is conceivable that immunotherapies might also be used in combination with other anti-addiction medications and could enhance behavioural therapies (Kosten et al 2005).

Active immunisation with drug-protein conjugate vaccines has been tested for cocaine, heroin, methamphetamine and nicotine in animals, and cocaine and nicotine vaccines are currently in clinical trials. In addition, the possibility of passive immunisation with high affinity monoclonal antibodies has been examined for cocaine, methamphetamine, nicotine and phencyclidine (PCP) in preclinical animal models. Current immunotherapies already show some efficacy, but improved antigen design and antibody engineering raise the hope for highly specific and rapidly developed treatments for both existing and future addictions (Kosten et al 2005).

1.8.1 “Anti-addiction” vaccines

Opiates

In 1974 Bonese *et al* provided the first proof of concept for the use of active vaccination in the treatment of drug dependence with a vaccine against heroin. However, this approach was abandoned due to concerns that individuals could easily switch to other opiates and thereby avoid the specificity of the anti-heroin antibodies. More recently, a vaccine against morphine/heroin has been shown, in rats, to result in high titres of antibodies showing equivalent specificity for both morphine and heroin. Structurally dissimilar opiate medications, such as buprenorphine, methadone and naltrexone, were not recognised, demonstrating the specificity of the antibodies. No major detrimental effects were detected, although there were some minor local injection reactions. Antibodies against heroin also appear to block its reinforcing effects in rodents, with rats failing to reacquire heroin-taking behaviour after immunisation against heroin, whereas self-administration was fully re-established in controls (Kosten et al 2005, Anton & Leff 2006).

Phencyclidine

Owens & Mayersohn (1986) conducted the first studies to demonstrate proof of concept for using passive immunisation in the treatment of drug abuse, showing that purified anti-phencyclidine (PCP) polyclonal antibodies could substantially alter the pharmacokinetics of PCP in dogs. This led to the development of haptens, which could stimulate antibodies capable of recognising a whole class of PCP-like drugs, rather than exclusively PCP (Owens et al 1988). Thus, if the

patient switched from one PCP-like designer drug to another, the therapy would still be effective (Kosten et al 2005). In rats, a single dose of anti-PCP mAb reduced the locomotor effects and brain concentrations of PCP in overdose studies (Owens & Mayersohn 1986, Valentine & Owens 1996, Valentine et al 1996, Proksch et al 2000, Hardin et al 2002, Laurenzana et al 2003a). This antibody profoundly reduced a wide range of pharmacological effects and significantly improved PCP-induced adverse health effects for up to 1 month after a single dose, even in the presence of occasionally lethal doses of PCP (Proksch et al 2000a, Hardin et al 2002, Laurenzana et al 2003a). The long functional half-life of the antibody suggests an important clinical role for passive immunisation in the treatment of PCP dependence, acute overdose, and longer-term excessive drug use.

Methamphetamine

Production of antibodies after immunisation with an anti-methamphetamine vaccine has been demonstrated in rats, but methamphetamine-induced locomotor effects were not attenuated as a result (Byrnes-Blake et al 2001). However, the same studies showed that administration of continuous high doses of methamphetamine during the entire course of immunisation had no effect on antibody production. This is important as it means that drug use during patient immunisation is unlikely to affect the production of antibodies. Passive immunisation with monoclonal antibodies against methamphetamine both reduced the dose of methamphetamine in the brain and slowed its rate of entry (Laurenzana et al 2003a&b, Byrnes-Blake et al 2003). These antibodies significantly attenuated locomotor activity in rats at low and moderate doses of methamphetamine, but not at doses, which significantly exceeded the antibody binding capacity. Self-administration of a low dose of methamphetamine was also completely blocked by the antibodies; however at higher unit doses of methamphetamine, rates of responding were increased, suggesting that the protective effects of the mAbs may be partially surmountable (Laurenzana et al 2003a&b, Byrnes-Blake et al 2003, Kosten & Owens 2005).

Cocaine

In rats, cocaine-specific antibodies from active or passive immunisation reduced early cocaine distribution to the brain and heart (by 25-80%) and decreased cocaine's locomotor, discriminative stimulus, and reinforcing behavioural effects. They also reduced the reinstatement of cocaine-seeking behaviour in a rat model of human relapse (Carrera et al 1995, 2000 & 2001, Fox et al 1996, Johnson & Ettinger 2000, Kantak et al 2000 & 2001). Phase I & II clinical trials of the Xenova anti-cocaine vaccine (TA-CD) have shown that it is safe and can produce antibodies with high specificity for cocaine (Kosten & Owens 2005). In phase II trials, cocaine dependent outpatients in the high-dose vaccination group developed higher antibody levels and used significantly less cocaine than those in the lower dose group (Martell et al 2005).

1.8.2 Anti-nicotine vaccines

In view of the low success rates achieved with current treatment options (see section 1.6.1) there is a clear need for improved pharmacotherapies for nicotine addiction. nAChRs are located throughout the brain and play an important role in "normal" neurotransmission within the CNS (see sections 1.3 & 1.4).

Targeting these receptors with agonists or antagonists, in an attempt to treat nicotine addiction, could interfere with normal CNS processes and result in a range of unwanted side effects. However, if the nicotine molecules could be prevented from stimulating the receptors in the first place, the reinforcing effects of nicotine might be attenuated.

Vaccination against nicotine results in the production of nicotine-specific antibodies, which circulate in the bloodstream. When nicotine enters the circulation upon smoking a cigarette, it is bound by the antibodies and retained in the serum, preventing it from entering the brain. This should result in a reduction in smoking-related reward and decrease the drive for continued tobacco consumption (Foulds et al 2004).

Currently there are at least 5 companies involved in the development of nicotine vaccines (Cytos Biotechnology, Nabi Biopharmaceuticals, Xenova Group Ltd., Chilka Ltd., and Independent Pharmaceutica AB) (Siu et al 2007). These vaccines differ mainly in their ligand chemistry, and have been explored by

various groups at the preclinical level, whilst several have also been examined in clinical trials (Hatsukami et al 2005, Maurer et al 2005).

All the vaccines have been shown to elicit an immune response, producing antibodies with high specificity for nicotine and low cross-reactivity with related compounds such as nicotine metabolites (typically <5%) (Maurer et al 2005, Hieda et al 1997, de Villiers et al 2002, Cerny et al 2002). In rats immunised against nicotine, 80-99% of nicotine in serum was bound to nicotine-specific antibodies, compared to less than 10% binding of nicotine to serum proteins in control rats (Hieda et al 1997, Pentel et al 2000, Keyler et al 2005, Satoskar et al 2003). As a consequence, brain nicotine levels were significantly reduced in vaccinated rats compared to controls (Cerny et al 2002, de Villiers et al 2004). Immunisation against nicotine has also been shown to attenuate nicotine-induced locomotor activity (Pentel et al 2000), reduce the incidence of seizures following a large dose of nicotine (Tuncok et al 2001), and prevent alleviation of nicotine abstinence syndrome through nicotine administration in rats (Malin et al 2001).

Initial clinical findings appear to show similar trends of nicotine vaccines towards efficacy in terms of safety and immunogenicity, and a greater percentage of patients were able to achieve abstinence from smoking in the highest dose vaccine group compared with the control group (Hatsukami et al 2005).

Anti-nicotine vaccines could be used for various indications: they may decrease the rewarding effects of nicotine in present smokers, reducing dependence and leading to higher likelihood of success in a cessation attempt. Former smokers could also benefit in that a “slip” during abstinence would have less of a rewarding effect and would therefore be less likely to lead to a complete relapse. Also, if administered to never smokers, the vaccine has the potential to curb the rewarding effects of nicotine on the brain of “experimenting smokers” (including young people), thus reducing the likelihood of the development of dependence. Finally, anti-nicotine immunotherapy has potential benefits for both pregnant smokers and the developing foetus, as nicotine (along with various other components of tobacco smoke) has been implicated as a neurotoxin in developing foetal tissues. A reduction in nicotine exposure to the brains of foetal rats, mediated by binding of nicotine to antibodies in the

maternal bloodstream, has been demonstrated. And with reductions in nicotine's reward-producing effects, vaccinated pregnant smokers could be more likely to attempt to quit and remain abstinent (Foulds et al 2004).

1.9 An anti-cotinine vaccine to aid smoking cessation

There are several potential problems associated with the use of anti-nicotine vaccines as a treatment for smoking cessation. Firstly, smokers who are finding it a challenge to remain abstinent might attempt to overcome the effects of the anti-nicotine antibodies by increasing their nicotine intake. This could potentially result in toxic effects associated with the consumption of a large dose of nicotine and would also lead to an increase in the exposure to various other toxins / carcinogens present in tobacco smoke (Stolerman et al 1991, Foulds et al 2004).

Blocking nicotine from acting on nAChRs by means of specific antibodies would also render all forms of NRT-based smoking cessation medication ineffective. These products are designed to relieve the withdrawal symptoms experienced by smokers during abstinence by providing an alternative source of nicotine without the additional toxins contained in tobacco smoke, and deliver the drug at a lower dose and slower rate than achieved through smoking, limiting the reinforcing effects and associated abuse potential in the process (see sections 1.5.1 & 1.6.1.1).

Cotinine has been shown to antagonise the actions of nicotine in vitro (see section 1.2.2.1). In clinical studies, cotinine has been reported to enhance withdrawal symptoms experienced by abstinent smokers, indicating that it may be antagonising the effects of nicotine on a functional level (Keenan et al 1994). In abstinent smokers treated with a nicotine or control patch, plus oral cotinine (80 mg) or placebo for 14 days, the group receiving cotinine reported no difference from placebo with respect to withdrawal symptoms, whereas a decrease in withdrawal symptoms was observed in the group receiving NRT. However, in combination with NRT cotinine appeared to antagonise the alleviating effects of nicotine (Hatsukami et al 1998).

Preclinical studies have suggested that high brain concentrations of cotinine (~0.3 μ M) may be achieved with chronic smoking since cotinine does not appear to be metabolised in the brain and shows slower outflow to the periphery relative to nicotine (Crooks et al 1997, Crooks & Dwoskin 1997, Ghosheh et al 1999). As a result, cotinine may limit the efficacy of nicotine replacement therapy (NRT) in promoting abstinence from smoking, likely through desensitisation of nAChRs. Therefore, if the effects of cotinine could be minimised or blocked, NRT might be more efficacious.

Immunisation with an anti-cotinine conjugate vaccine should elicit the production of cotinine-specific antibodies, which would bind any cotinine entering the bloodstream as a result of nicotine intake and subsequent metabolism, preventing it from passing into the brain. This should help to reduce the antagonism of nicotine's effects by cotinine.

Such a vaccine would be expected to increase the efficacy of NRT by enhancing the ability of nicotine to alleviate the withdrawal symptoms associated with nicotine abstinence, which are a major cause of relapse in abstinent smokers. Reduction of the antagonism of nicotine's actions by cotinine should also help to make the reduce-to-quit approach to smoking cessation a more viable option, as fewer cigarettes would need to be smoked to achieve the desired subjective effects.

1.9.1 Previous work on the anti-cotinine vaccine

This thesis continues the work begun as a previous PhD project (Pashmi 2004). With the help of collaborators, Pashmi synthesised several structural derivatives of cotinine for evaluation as potential anti-cotinine vaccines and established a basic immunisation regime.

In an initial trial, each of these derivatives was conjugated to bovine serum albumin (BSA), and rats were vaccinated with these conjugate vaccines. The levels of anti-cotinine antibodies raised in response to vaccination were examined. Of the cotinine derivatives tested, *trans*-4-thio-cotinine (CotSH) was found to produce the best immune response, and was therefore taken forward for further evaluation.

To maximise the immune response, and consequently the production of anti-cotinine antibodies, the carrier protein was switched from BSA to the more

immunogenic tetanus toxoid, giving TT-CotSH. This was found to further improve the antibody titres raised in response to immunisation.

The anti-cotinine antibodies obtained as part of these experiments were also evaluated in terms of their specificity for cotinine, compared to nicotine and a variety of other nicotine and cotinine metabolites. They were shown to be specific for cotinine with only very little cross-reactivity for norcotinine, a relatively minor metabolite of cotinine with no known pharmacological action (see section 1.2.2.3).

1.9.2 Aims of this PhD and layout of thesis

The aims of this PhD project were as follows:

- to confirm the hypothesis that cotinine could be acting as an antagonist to nicotine, using a model of striatal dopamine release
- to further characterise the immune response to anti-cotinine vaccination by measuring anti-cotinine and anti-tetanus toxoid antibody levels, as well as seeking to confirm the specificity of anti-cotinine antibodies
- to study whether immunisation against cotinine impacts on nicotine and cotinine distribution in the body
- to examine the effect of anti-cotinine vaccination on the upregulation of nicotine binding sites observed after prolonged nicotine exposure
- and finally, to use a wide range of behavioural measures of nicotine dependence to assess the effects of vaccination against cotinine in vivo.

The experiments described in this thesis were conducted as a series of 5 trials, with each consisting of an immunisation period (usually ~3 months) followed by behavioural experiments on the immunised animals and subsequent in vitro work on tissues and blood samples taken at the end of each trial. In the interest of clarity, this thesis has been subdivided into 3 results chapters based on experiments (rather than by trial), in order to facilitate the comparison of results from similar experiments between trials:

- In Chapter 2 the rationale for targeting cotinine in smoking cessation is examined, and the immune responses to anti-cotinine immunisation for each trial are summarised and compared.

- In Chapter 3 the impact of anti-cotinine immunisation on in vivo nicotine administration, assessed by in vitro measurements, are described. These experiments were carried out on tissue and blood samples taken from immunised and control rats at the end of each trial.
- Chapter 4 examines the effects of immunisation against cotinine on various behavioural measures relating to nicotine dependence.
- In the final discussion (Chapter 5), the results from each chapter are put into context, allowing overall conclusions to be drawn, and future avenues for research are indicated.

Chapter 2

Target validation & immune response

Chapter 2

Target validation & immune response

2.1 Introduction

Preclinical studies have shown that cotinine possesses weak activity at nicotinic acetylcholine receptors (nAChRs) and appears to antagonise the actions of nicotine both in vitro and in vivo (see Chapter 1) in a manner consistent with nAChR desensitisation. In clinical studies, cotinine has been reported to enhance withdrawal symptoms experienced by abstinent smokers (see Chapter 1), again indicating that it may be antagonising the effects of nicotine at a functional level. As high levels of cotinine are sustained in smokers, these data suggest that it might diminish the central effects of nicotine by desensitising nAChR.

Immunisation with an anti-cotinine conjugate vaccine should elicit the production of cotinine-specific antibodies, which would bind any cotinine entering the bloodstream as a result of nicotine intake and subsequent metabolism, and prevent it from entering the brain. This would reduce antagonism by cotinine in the CNS. Such a vaccine would enhance the efficacy of NRT with respect to alleviating withdrawal symptoms, which are a major cause of relapse in abstinent smokers.

In this chapter we studied the ability of cotinine to evoke the release of dopamine (DA) from the striatum of naïve rats in vitro, as well as comparing it with nicotine, to examine whether it might act as a (weak) agonist at nAChRs. The immune response induced by repeated vaccination of rats with the anti-cotinine conjugate vaccine was then characterised.

2.2 Dopamine release in naive rats

2.2.1 Introduction

The striatal and mesolimbic dopaminergic systems are implicated in the control of many important functions such as movement and posture, reward mechanisms, reinforcement, and motivational behaviours (Corrigall et al 1992 & 1994). Nicotine, like many drugs of abuse, activates the mesolimbic dopamine (DA) system (Nestler et al 2001, Mansvelder & McGehee 2002). Stimulation of nAChRs in the ventral tegmental area (VTA) leads to increased firing of DA neurons projecting from the VTA to the nucleus accumbens (NAcc) and other regions of the limbic forebrain, resulting in an increase of DA release in these areas (Benwell et al 1993, Nisell et al 1994, Stolerman & Shoaib 1991, Nestler 2001, Dani & Heinemann 1996, Picciotto et al 1998, Dani & De Biasi 2001).

This implies that nicotine exerts its effects on DA overflow by influencing impulse flow to the terminal field. Indeed, systemic injections of nicotine have been shown to increase burst firing of DA-secreting neurones in the VTA, and this effect is enhanced in animals pre-treated with nicotine (Nisell et al 1996). Several groups have shown that repeated administration of nicotine results in sensitisation of the mesolimbic DA system to the effects of the drug, a phenomenon that may be necessary for the development of drug dependence (Pierce & Kalivas 1997, Robinson & Berridge 2000, Ferrari et al 2001).

Accordingly, repeated nicotine administration results in sensitisation of locomotor stimulation and DA release in the NAcc (Ksir et al 1985, Benwell & Balfour 1992, Shim et al 2001) induced by a nicotine challenge. On the other hand, continuous infusion of nicotine has been observed to desensitise nAChRs, which mediate the DA release from neurons originating in the VTA (Pidoplichko et al 1997), and the DA overflow evoked by nicotine is substantially attenuated by the constant infusion of nicotine (Benwell et al 1995, Benwell & Balfour 1997)

Evidence that NAcc DA levels are important in reward has also come from VTA lesion studies and microperfusion of the NAcc with DA receptor antagonists, both of which lead to reduced self-administration of many addictive drugs, including nicotine, as well as a reduction in locomotor activity and diminished nicotinic binding in terminal areas (Stolerman & Shoaib 1991, Corrigall et al

1992 & 1994, Vezina et al 1994, Museo & Wise 1995, Louis & Clarke 1998, Balfour et al 2000).

DA release can be measured using a variety of techniques:

Microdialysis (and voltammetry) techniques enable the experimenter to examine DA overflow in awake, behaving animals, and can provide an opportunity to examine behavioural measures such as locomotor activity at the same time. Due to the high sensitivity and the small size of the probes used, it is possible to take measurements from distinct brain structures, providing good spatial resolution (Wonnacott et al 2002). The disadvantages are that this is a relatively invasive and technically demanding technique: a probe has to be inserted into the brain of each subject with great accuracy in order to ensure that release is being measured in the desired brain structure, making the procedure quite time- and work-intensive, and resulting in a low throughput. Microdialysis also measures DA overflow, rather than synaptic DA, and provides relatively low temporal resolution (although in vivo voltammetry can offer somewhat higher temporal resolution (Nisell et al 1997)). Drug concentrations reaching the nAChRs can only be estimated as they are frequently administered systemically, however local delivery of antagonists via the dialysis probe can help to define the site of action (Nisell et al 1994).

Continuous superfusion of brain slices or synaptosomes (Rapier et al 1990, Grady et al 1992) is a medium throughput assay, which allows the measurement of DA release over a period of time and in response to stimulation by agonists. However it is an in vitro technique, so behaviour and activity cannot be monitored in parallel. Superfusion of synaptosomes (Rapier et al 1990, Kaiser & Wonnacott 1998, Sharples et al 2000, Mogg et al 2004) only permits the experimenter to examine presynaptic effects, whereas brain slices (Dwoskin et al 1995, Sacaan et al 1995, Marshall et al 1996, Sershen et al 1997) maintain a reasonable level of local connectivity between individual neurons within a slice, making the preparation more representative of the intact brain. This allows some interaction to take place between the neurons within the terminal field, though not between the terminal field and the VTA. However, this can also complicate the interpretation of results due to indirect effects on transmitter release via synaptic connections between neurons within a slice.

Finally, the 96-well plate assay we chose to use in this thesis (Anderson et al 2000, Puttfarcken et al 2000, Jacobs et al 2002, Abin-Carriquiry et al 2006, Barik & Wonnacott 2006) is a simple and relatively high throughput technique, which maintains some limited connectivity within the brain minces used, compared to synaptosomes, and enables the measurement of DA release in response to agonist stimulation at a given time point compared to baseline release in response to buffer. It is less laborious than the commonly used superfusion method and permits the experimenter to simultaneously examine the effects of several compounds on transmitter release over a range of concentrations. Using this technique, several groups have characterised [^3H]DA and [^3H]NA release from a number of brain regions in the rat in response to nicotine and a variety of other nAChR agonists, as well as antagonists (Puttfarcken et al 2000, Anderson et al 2000, Mogg et al 2004, Cao et al 2005, Barik & Wonnacott 2006), while Abin-Carriquiry and colleagues have used the assay to compare the ability of several cytosine analogues to evoke striatal [^3H]DA and hippocampal [^3H]NA release (Abin-Carriquiry et al 2006).

Although nicotine-evoked DA release from minced nucleus accumbens (NAcc) in vitro has been described (Rowell et al 1987), the tiny size of this brain region precludes its use for most studies (Whiteaker et al 1995). We therefore chose to examine both nicotine- and cotinine-induced DA release from rat striatal minces (comprising dorsal striatum & NAcc).

2.2.2 Methods & Materials

Unless otherwise stated, chemicals were obtained from standard commercial sources.

2.2.2.1 Dopamine release from striatal slices

Assessment of [^3H]dopamine ([^3H]DA) release from striatal minces was performed using a 96 well assay (Anderson et al 2000, Jacobs et al 2002, Barik & Wonnacott 2006). For each experiment, 2 untreated rats were killed by cervical dislocation. Brains were rapidly removed and striata dissected and transferred to ice cold Krebs buffer (KB: NaCl 118 mM, KCl 2.4 mM, CaCl_2 2.4 mM, KH_2PO_4 1.2 mM, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1.2 mM, NaHCO_3 25 mM, D-glucose 10

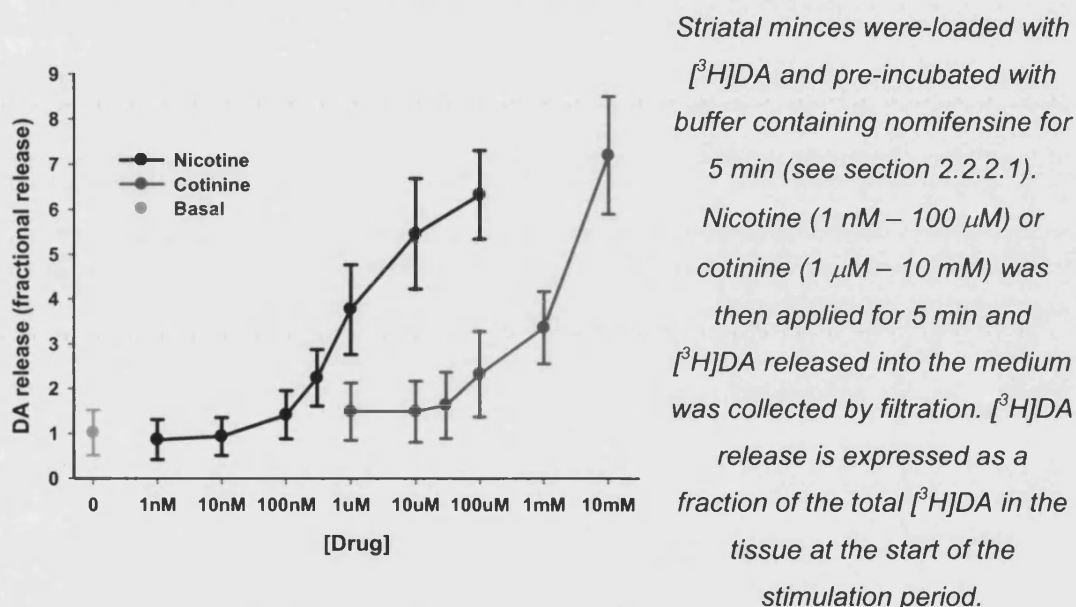
mM, ascorbic acid 1 mM; gassed with 95% O₂ and 5% CO₂ for at least 1 h at 37°C; pH adjusted to 7.4). Tissue was chopped 3 times (2 rotations at 60°) using a McIlwain tissue chopper to give prisms of 250 µm. Following 2 washes with warm KB, striatal slices were incubated for 30 min with 50 nM [³H]DA in 5 ml KB supplemented with 10 µM pargyline (to prevent [³H]dopamine degradation) at 37°C. To remove excess of tritium, 5 washes were performed over 25 min in KB containing 10 µM pargyline and 0.5 µM nomifensine (to prevent [³H]DA reuptake). Slices were then loaded onto a 96 well filter plate (model MABVN1250, Millipore, Hertfordshire, UK) and incubated at 37°C for 5 min with buffer (KB + pargyline + nomifensine) in the presence or absence of antagonist. Following this, buffer was removed by filtration (basal values) and collected in a 96-well Packard Optiplate™ (Perkin Elmer, Belgium). Buffer (70 µl) containing agonists and/or antagonists was then added to slices in each well (in each experiment, a buffer stimulation was included to determine the fractional release of [³H]DA in the presence of buffer alone). After a further 5 min at 37°C, buffer was collected by filtration in an Optiplate™ to determine the fraction of [³H]DA released. Microscint™ (170 µl per well) was added to optiplates and each well was counted for 1 min using a Microbeta liquid scintillation counter (Wallac 1450 Microbeta Trilux, Perkin Elmer, Finland), counting efficiency 30%. To determine the amount of tritium remaining in the slices, 30 µl HCl (0.1 M) was added to each well of the filter plate and incubated at 37°C for 1 h, before being centrifuged for 3 min at 1000 rpm with an optiplate to collect the filtrate. HCl was added to the wells again, and the process repeated twice. Microscint™ (170 µl per well) was added to the optiplate and each well was counted for 1 min as above. The amount of [³H]DA released was expressed as a percent of total radioactivity taken up in the slices prior to stimulation (i.e. amount of tritium released + tritium remaining in the tissue), giving a fractional release of [³H]DA.

2.2.3 Results

In order to confirm previous data obtained by Pashmi (2004), we examined the ability of cotinine to activate nAChR, using [³H]dopamine release from rat striatal minces as a well-defined system.

Nicotine (1 nM – 100 μ M) evoked the concentration-dependent release of [3 H]dopamine (see Figure 2.1), consistent with previous studies (Puttfarcken et al 2000, Grilli et al 2005, Abin-Carriquiry et al 2006). Roughly 1000-fold higher concentrations of cotinine (1 μ M – 10 mM) were required to elicit comparable levels of [3 H]dopamine release, which was also dependent on cotinine concentration. The estimated EC₅₀ values for nicotine and cotinine, assuming the maximal response had been reached, were 0.5-1.0 μ M and 0.5-1.0 mM, respectively.

Figure 2.1: Concentration dependence of nicotine- and cotinine-evoked [3 H]dopamine ([3 H]DA) release from rat striatal minces



Mean \pm S.E.M. (n = 3 independent experiments).

2.2.4 Discussion

To confirm that cotinine is able to activate nAChRs, we examined the ability of nicotine and cotinine to evoke [3 H]dopamine release from rat striatal minces. Application of each drug lead to the release of DA from striatal tissue in a concentration-dependent manner (see Figure 2.1), although approximately 1000-fold higher concentrations of cotinine were required to elicit comparable levels of release under the same conditions. Also indicated in Figure 2.1 is the basal 3 H outflow under buffer control conditions.

An important consideration in evaluating these data is the purity of commercial cotinine that is derived from nicotine: ~0.11% contamination with nicotine would

be sufficient to achieve the agonist potency observed. Importantly, GCMS analysis of the cotinine used throughout these studies found only 0.0022% impurity of nicotine present. This is insufficient to account for the shift in potency.

The estimated EC₅₀ values obtained here are slightly higher than those of ~380 nM (nicotine) and ~340 µM (cotinine) measured previously by Pashmi (Pashmi 2004). The EC₅₀ value for nicotine is also a little higher than that measured by others using the same 96-well technique (0.1-0.2 µM; Puttfarcken et al 2000, Abin-Carriquiry et al 2006), although it is within the range (0.1-4.0 µM) measured by groups utilising superfusion techniques (Grady et al 1992 & 1994, Sacaan et al 1995). Nicotine-induced striatal DA release is calcium dependent and can be inhibited by the nicotinic antagonists mecamylamine and DHβE using superfusion (Teng et al 1997, Grady et al 1992) and 96-well plate techniques (Puttfarcken et al 2000, Abin-Carriquiry et al 2006).

Cotinine has also been shown to act as an agonist at nAChRs, evoking DA release from rat striatal slices in a dose-dependent manner (Dwoskin et al 1999). However, cotinine displayed a much lower potency (EC₅₀ = 30 µM) than either nicotine or nornicotine (Dwoskin et al 1993 & 1999, Teng et al 1997). The EC₅₀ value for cotinine obtained by Dwoskin and colleagues is roughly 10-fold lower than that observed in our experiments, which may be due to differences in technique, as this group were measuring DA release from superfused striatal slices, whereas we used striatal minces in 96-well plates. Superfusion with a low calcium buffer or administration of the nicotinic antagonists mecamylamine or DHβE has been observed to inhibit cotinine-stimulated DA release (Dwoskin et al 1999, Pashmi 2004). Pashmi also found that pre-incubation of striatal minces with cotinine (100 µM – 1 mM) reduced DA release in response to nicotine, while Dwoskin and colleagues were able to show that desensitisation to the effect of cotinine occurs in superfused striatal slices, providing further evidence that cotinine acts at nAChRs to evoke DA release in these preparations (Dwoskin et al 1999, Pashmi 2004).

Targeting of cotinine with specific antibodies raised in response to vaccination with an anti-cotinine vaccine would remove the antagonism of nicotine's actions by cotinine, which should improve the efficacy of NRT.

2.3 Immune response

2.3.1 Introduction

In response to an antigen challenge, the immune system mounts a response, which involves the production of antibodies that recognise epitopes on the antigen and are specific for the particular antigen in question (reviewed in Chapter 1). The ability of a vaccine to elicit an immune response and initiate the production of specific antibodies is essential for the clinical effectiveness of the vaccine. This is also the case for therapeutic vaccines against drugs of abuse, including nicotine.

Numerous anti-nicotine vaccines are currently being developed (Hieda et al 1997, de Villiers et al 2002, Sanderson et al 2003, Cerny et al 2002, Maurer et al 2005, Meijler et al 2003), with several now being evaluated in clinical trials (Maurer et al 2005, Hatsukami et al 2005). Although the formulation varies between these vaccines, their mechanism of action is the same, as all are designed to target nicotine by initiating the production of antibodies that recognise nicotine (see Chapter 1). The antibodies bind nicotine when it enters the body and retain it in the bloodstream (Pentel et al 2000, Satoskar et al 2003, Cerny et al 2002, Keyler et al 2005, de Villiers et al 2004), preventing it from crossing the blood-brain-barrier and acting on nAChRs in the CNS. This leads to a reduction in the effects of nicotine administration (Pentel et al 2000, Malin et al 2001a & 2002). A possible problem with this approach is that it may lead smokers to attempt to compensate for the reduced rewarding effects of a cigarette by smoking more cigarettes or changing inhalation patterns in an attempt to maximise the amount of nicotine obtained from each cigarette. Also, it is likely to reduce the efficacy of NRT at relieving nicotine withdrawal syndrome in abstinent smokers during a quit attempt.

As discussed in the initial section of this chapter, the ability of cotinine to act as a weak agonist at nAChRs (see Figure 2.1), and the evidence that it can desensitise nAChRs (Vainio et al 1998b, Dwoskin et al 1999), make cotinine a possible target for the development of a new smoking cessation therapy.

Vaccination against cotinine should lead to the production of anti-cotinine antibodies that could bind the metabolite and prevent it from acting on nAChRs in the brain, thus effectively removing the cotinine-mediated antagonism of nicotine's actions. This should make NRT more efficacious, as well as allowing

a smoker to decrease the number of cigarettes smoked as each one would have a greater effect. Such a vaccine could therefore be used to assist with a smoking cessation attempt in conjunction with NRT, but also in a reduce-to-quit approach to smoking cessation.

In previous work relating to this anti-cotinine vaccine, Ghazaleh Pashmi evaluated several cotinine derivatives conjugated to bovine serum albumin (BSA) in terms of their immunogenicity in rats (Pashmi 2004). Trans-4-thio-cotinine (CotSH) was found to be the most effective derivative of cotinine for eliciting the production of anti-cotinine antibodies, and was therefore taken forward for further studies. Conjugation of this derivative to tetanus toxoid (TT-CotSH) instead of BSA further increased the immune response.

In this project, 5 trials (see table in section 2.3.2.3 for an overview) were undertaken at Bath (Trials 1 – 3) and Newcastle-upon-Tyne (Trials 4 & 5). The immunisation regimes for each trial are described in section 2.3.2.3, while neurochemical and behavioural experiments performed as part of these trials are recorded in Chapters 3 & 4.

As a starting point for the work described in this thesis, we further optimised the anti-cotinine vaccine by examining the immune response to vaccination with TT-CotSH in conjunction with several different adjuvants/adjuvant combinations (Trial 1). Adjuvants are a critical factor in determining the efficacy of a vaccine since they help to maximise the immune response upon immunisation (see Chapter 1). In this chapter, anti-cotinine antibody titres achieved in all trials undertaken are compared, and the development of anti-tetanus toxoid antibody titres was also monitored and compared across trials. In addition, we sought to confirm the specificity of antibodies raised against TT-CotSH for cotinine, as observed by Pashmi (2004).

2.3.2 Methods & Materials

Unless otherwise stated, chemicals were obtained from standard commercial sources.

2.3.2.1 Vaccine preparation

2.3.2.1.1 Synthesis of *trans*-4-thio-cotinine (CotSH)

Synthesis of the cotinine derivative (CotSH) was originally carried out as part of a previous PhD project (G. Pashmi) by Professor Tim Gallagher and Dr Sharn Ramaya at the University of Bristol in 2001. All experiments carried out at the University of Bath as part of this thesis were conducted using conjugates made from this batch of derivative. In 2006 Dr Stephen Husbands (University of Bath) was asked to synthesise a fresh batch of CotSH, due to concerns over the age of the original derivative. Conjugates from this derivative were used in the behavioural experiments performed at the University of Newcastle (Trials 4 & 5).

(±)-*trans*-4-Cotinine carboxylic acid was employed for attachment of a sulphhydryl group. The reaction was conducted under nitrogen, and all glassware was pre-dried in an oven at 150°C for 12 h prior to use. (±)-*trans*-4-Cotinine carboxylic acid (200 mg, 0.9 mmol), 2-aminoethanethiol.HCl (103 mg, 0.9 mmol) and triethylamine (0.13 ml, 1.8 mmol) were dissolved in dry dimethylformamide (DMF; 1.5 ml). The reaction mixture was cooled to 0°C in an ice bath, and EDCI.HCl (174 mg, 0.9 mmol) was then added. The reaction mixture was warmed to room temperature and stirred for a further 12 h. The solvent was removed *in vacuo*, and water (5 ml) and CH₂Cl₂ (5 ml) were added to the residue. The aqueous phase was washed with CH₂Cl₂ (4 x 5 ml). The combined organic extracts were dried over Na₂SO₄, filtered, and the solvent removed *in vacuo*. Purification by flash chromatography, using 5 % methanol / dichloromethane as eluent, gave the product (136 mg, 54 % yield) as a colourless oil.

[Found; (M⁺), 279.1045. C₁₃H₁₇O₂N₃S requires (M⁺) 279.1041]; δ_H (400MHz, CDCl₃) 8.58 (1 H, d d, *J* 4.9), 8.50 (1 H, d, *J* 2.0), 7.60 (1 H, d t, *J* 2.0, 7.8), 7.38 (1 H, d d, *J* 4.9, 7.8), 6.77 (1 H, br t, *J* 5.4), 4.79 (1 H, d, *J* 6.8), 3.51-3.35 (2 H, m), 2.92-2.68 (5H, m), 2.64 (3 H, s, CH₃), 1.32 (1 H, t, *J* 8.3, SH); ν_{max} (thin film)/cm⁻¹ 3500-3100 (br NH), 2543 (S-H), 1684 (C=O); *m/z* (EI⁺) 279 [M⁺, 65%].

The product is racemic.

2.3.2.1.2 Conjugation to Tetanus Toxoid

CotSH was conjugated to tetanus toxoid (TT) using an NHS ester-maleimide heterobifunctional cross-linker. TT was activated with S-GMBS through its amino groups: TT (5 mg/ml in 100 mM sodium phosphate buffer pH 7.2) was stirred at room temperature with an 80-fold molar excess of SGMBS (Pierce) for 2 h. The maleimide-activated TT was then purified by applying the reaction mixture to a desalting column (PD-10 desalting column, Amersham Pharmacia Biotech) to remove excess reagent and reaction by-products. Fractions (1 ml) were collected using 100 mM sodium phosphate buffer (pH 6.8) to elute the activated TT. Fractions containing TT, determined by measurement of absorbance at 280 nm, were pooled and the protein concentration was measured by the Lowry method (Lowry 1951). The number of activated sites on the TT molecules was then determined using Ellman's assay (Hermanson 1996).

CotSH was added to the activated TT in a 2:1 ratio (mole:mole) of CotSH/number of maleimide functions, and incubated for 1 h at room temperature under agitation (TT-CotSH). Cysteine (4 mg/ml in sodium phosphate buffer 100 mM, pH 6.8) was then added to quench any unattached sites present on TT-CotSH, and incubated for 30 min at room temperature under agitation. Conjugate solutions were dialysed against sodium phosphate buffer (2 mM, pH 6.8) overnight (4 °C), filtered using a millex filter (0.22 µm) in a sterile environment, and stored at 4 °C until use. Control vaccine was prepared by coupling cysteine to TT (TT-Cysteine), instead of CotSH. Conjugates were dialysed against phosphate buffer (2 mM, pH 6.8) for 2 h using Slide A-lyzer discs (3500MWCO - Pierce) with 3 buffer changes.

Lowry's and Ellman's assays were conducted to determine the number of activated sites still remaining. Comparison of the number of maleimide functions before and after quenching determined the number of CotSH attachments per TT molecule (typically 8 – 14).

After the conjugations for Trial 1, which I carried out under the supervision of staff at GSK Biologicals in Rixensart, Belgium, all subsequent conjugates were produced there and then shipped to me at Bath / Newcastle. A fresh batch of conjugates (TT-CotSH and TT-Cysteine) was produced for each trial. Using the fresh batch of derivatives synthesised in 2006, new conjugates (TT-CotSH and

TT-Cysteine) were produced for Trials 4 & 5. Unfortunately, it appears that the vials containing these new conjugates were mislabelled, i.e. TT-CotSH labelled “TT-Cysteine”, and vice versa (for more details see section 2.3.4).

2.3.2.1.3 Formulation of vaccine

Conjugate solutions were mixed with AS02v adjuvant in a 1:1 ratio. In the adjuvant optimisation study (Trial 1) other adjuvants were also used; these were coded (X, Y & Z) on request of GSK (see section 2.3.2.3.1). AS02v, a GSK proprietary adjuvant system, is an oil-in-water emulsion-based adjuvant system containing MPL and QS21. AS02v has been shown to elicit a high immune response (unpublished).

2.3.2.2 Animals

For all behavioural experiments carried out at the University of Newcastle upon Tyne (see sections 2.2.3.4, 2.2.3.5, and Chapter 4), male Hooded Lister rats (Harlan UK) were used. They were housed in rooms maintained at 20-22 °C, under a 12:12 h light:dark cycle (lights on 0700-1900 h). Rats had access to food and water *ad libitum*, except for those involved in the taste aversion experiments (see section 4.4), which had restricted access to water.

Rats used in the intravenous self-administration experiments were housed in pairs; all others were housed in groups of 4. For the conditioned taste aversion experiments, rats were housed individually and allowed to acclimatise for a week prior to the experiment. After completing the taste aversion procedures, rats were re-housed in their original groups of 4 for all further procedures (locomotor activity, additional immunisation and mecamlamine precipitated withdrawal).

All experiments carried out at the University of Bath utilised male Sprague-Dawley rats, 6-7 weeks old at the start of the immunisation regime, which were obtained from the University of Bath breeding colony or Charles River UK, and housed in groups of 3-4 with access to food and water *ad libitum* in rooms maintained at 22 ± 2 °C under a 12:12 h light:dark cycle (lights on 0700-1900 h).

All studies complied with local and national ethical requirements, and were carried out according to the Animals (Scientific Procedures) Act of 1986 under license from the UK Home Office.

2.3.2.3 General immunisation regime and overview of trials

The immunisation schedule used was taken from Hieda *et al* (2000) with variations. Rats were immunised (i.m.) with TT-CotSH (5 µg) or TT-Cysteine (5 µg) in a final volume of 250 µl (125 µl per leg, i.m.), typically on days 0, 21 and 35. TT-Cysteine was used to control for effects of immunisation with the carrier protein. In order to maintain antibody titres, rats were boosted at regular intervals, typically on days 63 and 77. Blood samples were also taken at immunisation time points (and between immunisations in some studies) in order to monitor the development of antibody titres (see section 2.2.4).

The following table gives an overview of the experiments carried out as part of each of the 5 trials, in addition to the series of immunisations:

| Trial No | Location | Experiments carried out |
|-----------------|-----------------|--|
| 1 | Bath | Adjuvant comparison (Chapter 2) Trial run of 5-day locomotor experiments (Chapter 4) |
| 2 | Bath | 5-day locomotor experiments (Chapter 4) Radioligand binding (Chapter 3) |
| 3 | Bath | 12-day locomotor experiments (Chapter 4) Radioligand binding (Chapter 3) Blood / brain nicotine & cotinine levels (Chapter 3) |
| 4 | Newcastle | Intravenous self-administration (Chapter 4) |
| 5 | Newcastle | Conditioned taste aversion (Chapter 4) Acute locomotor experiments (Chapter 4) Mecamylamine-precipitated withdrawal (Chapter 4) Radioligand binding (Chapter 3) Blood / brain nicotine & cotinine levels (Chapter 3) |

Immunisation schedules for each individual trial are given in more detail below.

2.3.2.3.1 Trial 1 – Comparison of adjuvants (Bath)

40 male Sprague-Dawley rats (6-7 weeks old) were divided into four groups of 10 animals, each of which received the conjugates with a different adjuvant treatment (adjuvant X, adjuvant Y, adjuvant Z or AS02v). Half of each group (5 rats) was immunised with the active conjugate (TT-CotSH), the other half with the control (TT-Cysteine).

Timeline:

Day 0 – Bleed & immunisation
Day 21 – Bleed & immunisation
Day 35 – Bleed & immunisation
Day 49 – Bleed
Day 63 – Bleed & immunisation
Day 77 – Bleed

After completion of the initial immunisation schedule, rats immunised with conjugates plus AS02v were given an additional booster immunisation. All other groups were used for a trial run of locomotor experiments.

Adjuvants X, Y & Z:

Day 98 – Bleed
Day 101-105 – Locomotor activity experiments
Day 105 – Cull (blood & brain collection)

AS02v:

Day 110 – Bleed & immunisation
Day 115 – 118: Cull & final bleed

2.3.2.3.2 Trial 2 – 5-day conditioned locomotor experiments (Bath)

96 male Sprague-Dawley rats (6-7 weeks old) were divided into 8 groups with 12 rats per group. 48 rats were immunised with TT-CotSH plus AS02v, the other 48 with the TT-Cysteine control conjugate plus AS02v. Half the rats from each group started their immunisation regime a week after the other half; staggering of immunisations and locomotor experiments by a week was necessary due to limits on the equipment available.

Timeline:

Day 0 – Bleed & immunisation
Day 21 – Bleed & immunisation
Day 35 – Bleed & immunisation
Day 63 – Bleed
Day 91 – Bleed & immunisation
Day 105 – Bleed & immunisation
Day 119 – 124 – Locomotor activity experiments
Day 124 – Cull (Blood & brain collection)

2.3.2.3.3 Trial 3 – 12-day locomotor experiments (Bath)

48 male Sprague-Dawley rats (6-7 weeks old) were divided into 4 groups with 12 rats per group. Half the rats were immunised with TT-CotSH plus AS02v, the other half with the TT-Cysteine control conjugate plus AS02v.

Upon completion of the locomotor experiments, half the animals were culled immediately. The remaining half was given further daily injections of nicotine or saline and culled 5 min after their final injection.

Timeline:

Day 0 – Bleed & immunisation

Day 21 – Bleed & immunisation

Day 35 – Bleed & immunisation

Day 49 – Bleed

Day 63 – Bleed & immunisation

Day 77 – Bleed & immunisation

Day 91 – 103 – Locomotor activity experiments

Day 103 – Cull 1st half (blood sample collection)

Day 103 – 106 – Additional nicotine/saline injections (once daily)

Day 106 – Cull 2nd half (blood & brain collection)

2.3.2.3.4 Mislabelling of conjugates

The final two trials were undertaken in Newcastle. Newly prepared conjugates, produced at GSK Rixensart, were received after the start of these trials and all immunisations were then switched to the new conjugates. As immunisation was staggered, some rats' immunisation schedule had already been started, using the old conjugates. This meant that due to the mislabelling of the vials, control rats were unintentionally immunised with the active conjugate (TT-CotSH), and rats supposedly receiving the active conjugate were immunised with the control (TT-Cysteine) instead. As a result, control rats in Trials 4 & 5 started to raise antibodies against cotinine.

In an attempt to restore active and control groups in Trial 5, rats were given an extra large booster dose (25 µg instead of the usual 5 µg) of "old" conjugates (i.e. conjugates used at the start of the trial before the fresh batch arrived).

2.3.2.3.5 Trial 4 – Intravenous self-administration (Newcastle)

Surgery to implant the intravenous catheter for self-administration experiments was performed on a total of 58 male hooded Lister rats. Of an initial 37 animals,

one was culled because of infection post-surgery. In order to replace rats, which had been excluded due to blocked catheters and/or lack of acquisition of intravenous self-administration, a further 21 animals were implanted with catheters (2 were culled post-surgery due to a blocked catheter and infection, respectively). In all, a total of 55 rats proceeded to the acquisition phase. Of these, only 18 satisfied the criteria for stable maintenance and completed the immunisation schedule (5 injections of TT-CotSH / TT-Cysteine plus adjuvant AS02v). A further 3 rats were lost during the extinction and reinstatement phases, leaving 15 rats which completed the entire series of self-administration experiments.

Timeline:

Surgery & Recovery (10 – 14 days)

Acquisition (21 – 132 sessions)

Maintenance (~65 sessions (average), including immunisation period)

Day 0 – Bleed & immunisation

Day 21 – Bleed & immunisation

Day 35 – Bleed & immunisation

Day 63 – Bleed & immunisation

Day 77 – Bleed & immunisation

Extinction (3 – 17 sessions)

Reinstatement (~30 – 55 sessions)

Cull (blood sample collection)

**2.3.2.3.6 Trial 5 – Conditioned taste aversion / locomotor activity /
mecamylamine-precipitated withdrawal (Newcastle)**

48 male hooded Lister rats (6-7 weeks old) were used in this study. Half the rats were immunised with TT-CotSH plus AS02v, the other half with the TT-Cysteine control conjugate plus AS02v.

Timeline:

Day 0 – Bleed & immunisation

Day 21 – Bleed & immunisation

Day 35 – Bleed & immunisation

Day 63 – Bleed & immunisation

Day 77 – Bleed & immunisation

Day 89 – 113 – Conditioned taste aversion

Day 119 – 124 – Locomotor experiments

Day 125 – Bleed & immunisation (large dose)

Day 138 – 158 – Mecamylamine-precipitated withdrawal (staggered)

Cull (blood & brain collection)

Once the initial immunisation schedule had been completed, 32 of the 48 rats were used in the conditioned taste aversion and locomotor procedures. The other 16 rats were kept as nicotine-naïve controls for the mecamylamine-precipitated withdrawal experiments.

Due to the mislabelling of a batch of conjugates (see section 2.2.1.2.1), rats immunised with "control" vaccine had started to develop anti-CotSH titres, and the titres of those receiving "active" vaccine were much lower than anticipated. For this reason, all 48 rats received a further extra large booster immunisation (25 µg conjugate per rat instead of the usual 5 µg, plus AS02v; see section 2.2.1.2.1) once the locomotor experiments had been completed.

In order to maximise the chance of seeing a vaccine effect in the behavioural tests in this trial (mecamylamine-precipitated withdrawal), we selected only those rats with the lowest and highest anti-CotSH titres for control and active groups, respectively. The idea of having nicotine-naïve control groups was abandoned as a result, and a mixture of nicotine-naïve animals and rats with a history of nicotine treatment (previously used in conditioned taste aversion and locomotor experiments) was consequently divided into treatment groups for the withdrawal experiments.

2.3.2.4 Blood sampling & treatment

Blood samples (~300 µl) taken from the lateral tail vein (under isoflurane anaesthesia) at vaccination time points and at sacrifice (mainly trunk blood) were centrifuged at 4 °C, 1500 x g, for 30 min, and the supernatant centrifuged again. Aliquots of sera were stored at -20 °C. These samples were used to monitor antibody titres by ELISA (see section 2.2.5), and to determine blood nicotine and cotinine levels as a result of treatment with nicotine (see section 3.2).

2.3.2.5 ELISA Methods

2.3.2.5.1 ELISA for measuring anti-CotSH titres

Maxisorp 96 well plates were coated with CotSH (1 µg/ml in carbonate buffer (1 carbonate-bicarbonate buffer capsule in 100ml deionised water); 50 µl per well) overnight at 4 °C. Plates were incubated with saturation buffer (phosphate buffered saline, pH 7.4, Tween-20 (0.1 % v/v), deactivated foetal bovine serum

(4 % w/v), bovine serum albumin (1 % w/v)) plus 1 % w/v casein (100 µl/well, pH 7.4) for 1 h at 37 °C, before being washed 3 times with PBS-Tween (0.1 %). Sera from immunised rats were serially diluted (1:2 times each time) in saturation buffer, and 50 µl of each dilution added to the plate and incubated at 37 °C for 1 h. Plates were washed again, and biotinylated anti-rat Ig from sheep (1:3000 in saturation buffer) was added (50 µl/well). Plates were incubated for 2 h at 37 °C, then washed and incubated with streptavidin-biotinylated HRP (1:1000 in saturation buffer, 50 µl/well) for 30 min at 37 °C. After washing, plates were incubated with TMB peroxidase (100 µl/well) for 10 min in the dark. Sulphuric acid was added (0.4 N, 50 µl/well) and absorbance measured at 450 nm and 620 nm, using a plate reader. Sera with known titre levels (from previous trials) were used as controls.

2.3.2.5.2 ELISA for measuring anti-TT titres

For the anti-TT ELISA, plates were coated with TT-cysteine (1 µg/ml in carbonate buffer), and the ELISA performed as above. Sera with known titre levels (from previous trials) were used as controls.

2.3.2.5.3 Competitive ELISA

For the competitive ELISA, sera were incubated with 7 dilutions of competitors (1 mM – 1 nM) at 4 °C overnight. The competitors used were nicotine and cotinine, plus 5 further metabolites of nicotine: nornicotine, norcotinine, trans-3-hydroxycotinine, nicotine-1-oxide and cotinine-N-oxide (purchased by GSK). An ELISA was then performed as above, but using plates coated with 0.05 µg/ml CotSH, and adding 100 µl of the sera-competitor solutions in duplicate instead of serial dilutions of sera. Sera (from rats immunised with active and control vaccine, respectively), and incubated with saturation buffer alone were used as controls.

2.3.2.6 Data analysis

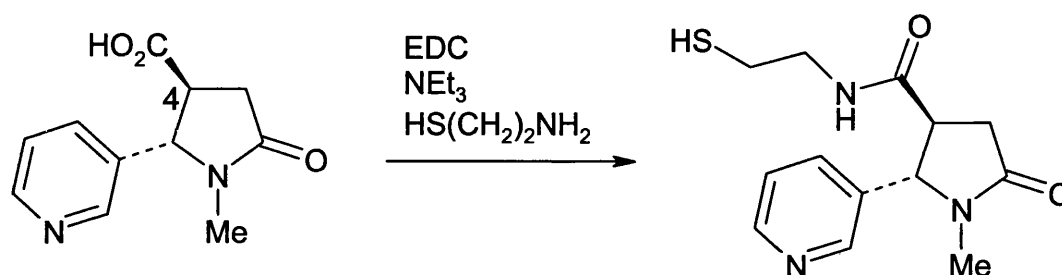
Antibody titres are expressed as the dilution producing a 50% maximal optical density on ELISA (i.e. mid-point titres), and data shown as means ± S.E.M.

2.3.3 Results

2.3.3.1 Chemistry, vaccine formulation & overall safety profile

Cotinine was chemically modified to introduce a thiol group attached to an amide spacer via the C4 position of cotinine (see Figure 2.2) to give *trans*-4-thiol cotinine (CotSH). This was coupled to the carrier protein tetanus toxoid (TT) prior to vaccination, using an NHS ester-maleimide heterobifunctional cross-linker, which resulted in the attachment of 8 – 14 molecules of derivative per TT molecule. Cysteine conjugated to TT was used as the control vaccine, with similar levels of conjugation achieved.

Figure 2.2: Synthesis of *trans*-4-thio-cotinine (CotSH) from (\pm)-*trans*-4-cotinine carboxylic acid



A thio group, attached to an amide spacer, was introduced into *trans*-4-cotinine by reaction of *trans*-4-cotinine carboxylic acid with 2-aminoethanethiol and triethylamine in DMF (see section 2.3.2.1.1).

Safety of vaccination & blood sampling

Rats were immunised repeatedly with TT-CotSH or TT-Cysteine plus one of four adjuvants/adjuvant combinations, and blood samples taken at regular intervals to monitor the development of antibody titres. Vaccination with either conjugate plus the various adjuvants/adjuvant combinations appeared to be safe and well tolerated. No adverse reactions to the vaccines or adjuvants were observed in any of the rats.

In order to minimise irritation of the tail, rats were bled alternately from the left and right lateral tail veins. With the exception of (very rare) minor bruising to the tail the following day, no adverse reactions to the bleeding protocol were observed and none of the rats appeared to be in any pain or discomfort as a result of the procedure.

2.3.3.2 Anti-CotSH titres: General immune response

No anti-cotinine antibodies were detected in any of the rats prior to the start of vaccinations (in any of the trials). Repeated vaccination with TT-CotSH resulted in the production of anti-cotinine antibodies. These could be detected by day 21, however at this point the levels were often still too low to determine mid-point titres. Antibody titres increased with each vaccination; however titres tended to wane over time and booster injections were required in order to maintain antibody levels (see below).

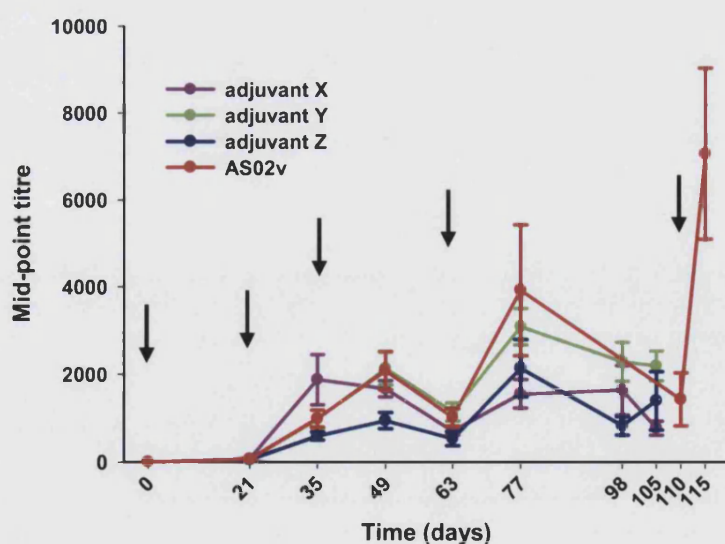
TT-Cysteine did not produce any titres in the anti-CotSH ELISA, consistent with the specificity of the method and the absence of cross-reacting antibodies after immunisation with TT-Cysteine.

2.3.3.3 Anti-CotSH titres: individual trials

Trial 1 (Adjuvant comparison)

Mid-point titres of anti-cotinine antibodies gradually increased in all groups vaccinated with TT-CotSH (see Figure 2.3).

Figure 2.3: Immune response to anti-cotinine vaccination (Trial 1)



Rats were immunised with 5 μ g TT-CotSH, plus one of 4 adjuvants/ adjuvant combinations, on the days indicated (arrows). Blood samples were collected at each timepoint and serum mid-point titres of vaccinated rats ($n = 5$) were determined by ELISA, using trans-4-thiol cotinine coated plates.

Antibody titres are expressed as the dilution producing 50% maximal optical density on ELISA (i.e. mid-point titres).

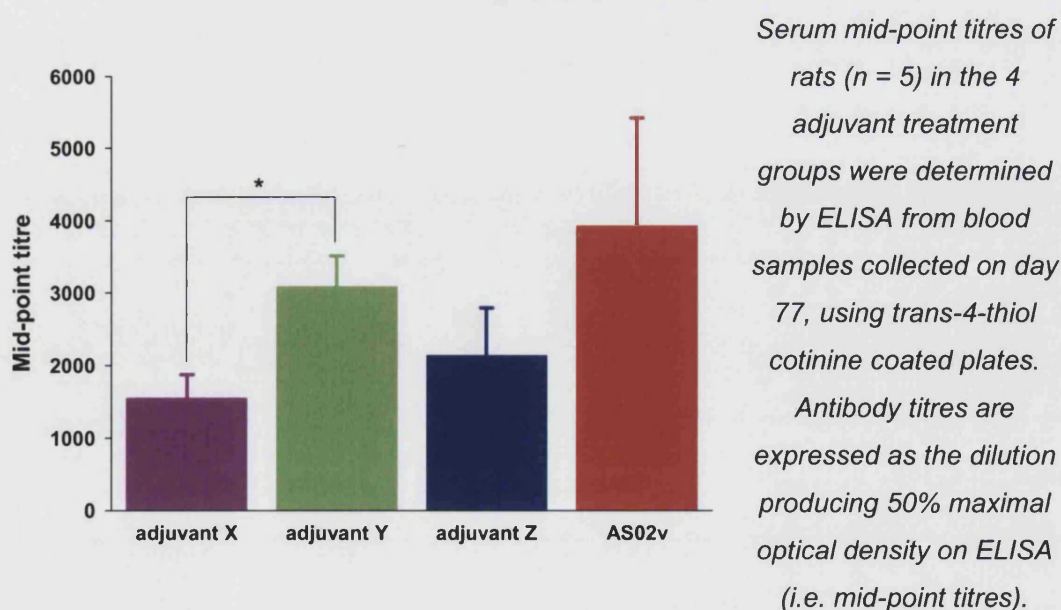
Data presented are means \pm S.E.M.

By day 77, after 3 initial injections (day 0, 21 & 35) and one booster dose (day 63), a comparison of anti-cotinine antibody levels between adjuvant treatment groups revealed that the mean mid-point titre of the group treated with AS02v was slightly greater than the other treatment groups (see Figure 2.4), however this was not significant. Treatment with adjuvant Y produced significantly higher anti-cotinine antibody levels than adjuvant X ($1:3093 \pm 420$ and $1:1552 \pm 324$, respectively; $p=0.0197$), however the mean mid-point titre for both these groups, and that for adjuvant Z ($1:2140 \pm 655$), was lower than that for AS02v ($1:3926 \pm 1500$). AS02v was therefore used as an adjuvant in all subsequent trials (see Figure 2.9 for comparison of titres between trials).

The AS02v-treated group was given another booster injection on day 110 and anti-cotinine titres measured again. The final booster resulted in a marked increase in antibody levels to $1:7061 \pm 1973$ (see Figure 2.3), so in subsequent trials 2 booster injections were given prior to behavioural experiments.

Rats from the other three adjuvant groups (X, Y & Z) were used for a vaccinated pre-trial of the conditioned locomotor experiments (see section 4.2.2.3). Their titres were monitored by taking blood samples prior to behavioural experiments, and when they were culled, by which time anti-cotinine antibody levels had decreased slightly in these groups (see Figure 2.3).

Figure 2.4: Immune response to anti-cotinine vaccination – comparison of adjuvants

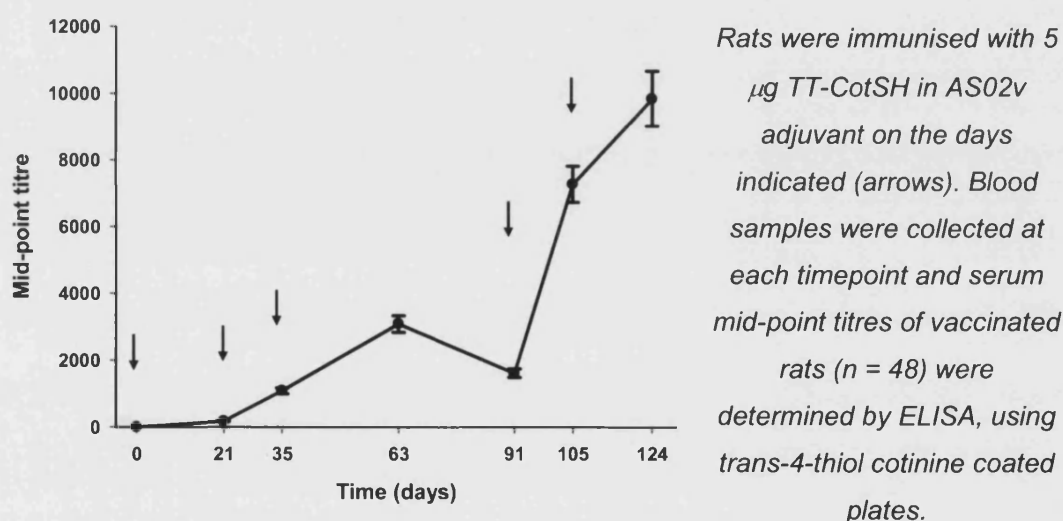


Data presented are means \pm S.E.M.; * $p < 0.05$

Trial 2

Anti-cotinine titres in rats immunised with TT-CotSH plus AS02v increased from one immunisation to the next, and had reached similar levels ($1:7266 \pm 545$) to those achieved in Trial 1 (AS02v group) by day 105 (see Figure 2.5 & 2.9), when rats were given a final booster injection. Blood samples obtained at the end of the experiment (day 124) revealed that antibody levels had increased even further ($1:9832 \pm 826$) over the course of the locomotor experiments (see section 4.2.2.4). There were no significant differences in post-locomotor titres between locomotor treatment groups, i.e. no effects of drug or pairing (data not shown).

Figure 2.5: Immune response to anti-cotinine vaccination (Trial 2)

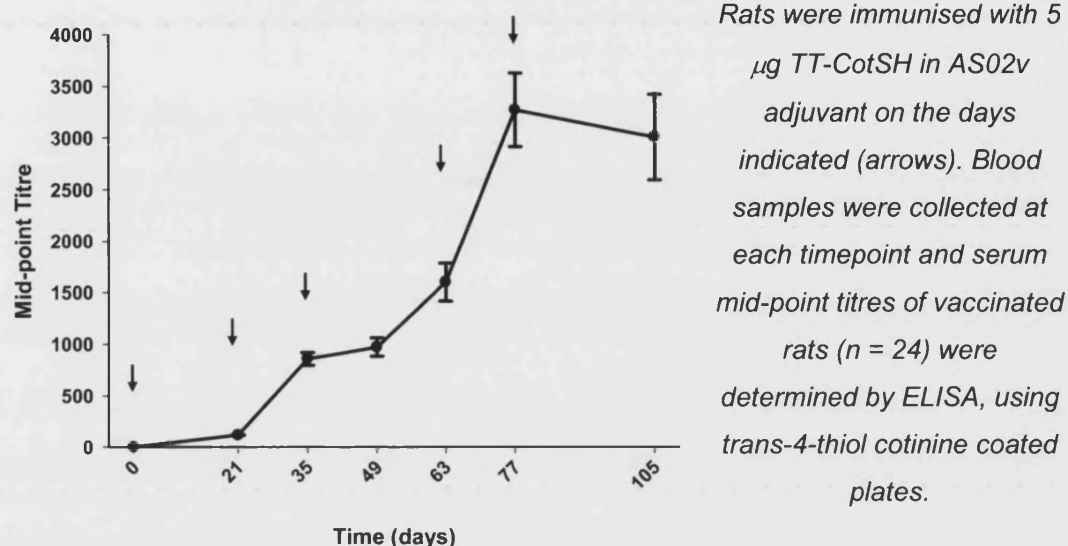


Antibody titres are expressed as the dilution producing 50% maximal optical density on ELISA (i.e. mid-point titres).

Data presented are means \pm S.E.M.

Trial 3

The anti-cotinine antibody titres achieved in rats immunised with TT-CotSH plus AS02v increased from one immunisation to the next over the course of Trial 3 to a maximum of $1:3272 \pm 355$, but were markedly lower (see Figure 2.6) than those obtained in Trial 2 (see Fig 2.9). This might have been due to the age of the cotinine derivative (~3-4 years), which prompted the decision to produce a fresh batch of CotSH from which the conjugate for Trials 4 & 5 could be made.

Figure 2.6: Immune response to anti-cotinine vaccination (Trial 3)

Antibody titres are expressed as the dilution producing 50% maximal optical density on ELISA (i.e. mid-point titres).

Data presented are means \pm S.E.M.

Trial 4

Anti-cotinine antibody titres obtained in Trial 4 are summarised in Figure 2.8. Rats entered the vaccination phase of the experiment individually once they had fulfilled stability criteria for self-administration behaviour (see section 4.3.2.5). A couple of weeks after the first rat had received its first vaccination, the new batch of conjugates arrived from GSK Rixensart, and all subsequent injections consisted of these new conjugates. This meant that while some rats received an injection of "old" conjugate (and one rat was given 2 injections of "old" conjugate), approximately half the animals were only vaccinated with "new" conjugates, as they did not fulfil stability criteria until after the switchover point. Due to the mislabelling of the vials containing the fresh conjugates, rats were effectively mis-vaccinated, resulting in 3 categories of treatment groups based on vaccination history.

(For both Trial 4 and Trial 5, "active" & "control" groups are used here to define the original treatment the rats were assigned to, prior to the mislabelling of vials becoming apparent, whereas active & control (without inverted commas) refer to the final groups based on antibody titres raised.)

- "Active" & "control" groups that had only been injected with "new" conjugates: While vaccination entirely with "new" TT-Cysteine resulted in measurable anti-cotinine antibody levels from day 21 onwards (Figure 2.7), rats injected with "new" TT-CotSH throughout raised no measurable anti-cotinine titres whatsoever. Based on these findings it was clear that the conjugate vials must have been mis-labelled (i.e. swapped).

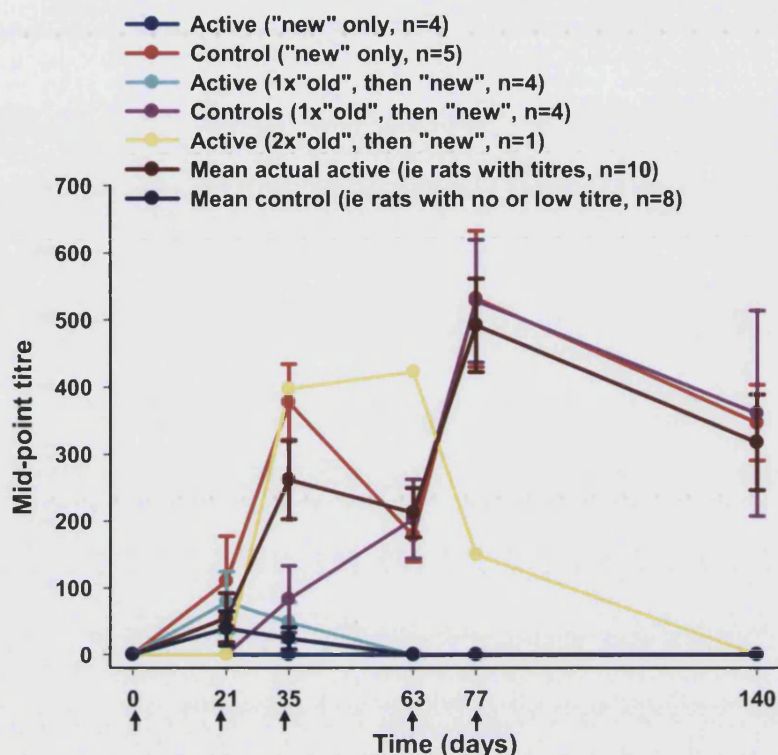
- "Active" & "control" groups that had been given an initial injection of "old" conjugates, but had received "new" conjugates from the second vaccination onwards:

Rats given one injection of "old" TT-CotSH exhibited initial antibody titres at day 21 (Figure 2.7), however these diminished again very quickly, as the "new" TT-CotSH administered thereafter was actually the control conjugate (TT-Cysteine). Those animals initially vaccinated with "old" TT-Cysteine on one occasion, did not at first produce measurable anti-cotinine antibodies, however after repeated injections of "new" TT-Cysteine, antibody titres rose to similar levels as seen in the rats that had received "new" control conjugate (actually TT-CotSH) only.

- One rat that was vaccinated with "old" conjugate (TT-CotSH, i.e. active) twice before the switch to the "new" conjugate took place:

At first this rat developed titres similar to the group vaccinated entirely with "new" TT-Cysteine (Figure 2.7), however, after being switched to injections of "new" TT-CotSH, antibody production began to level off and decreased progressively until levels were undetectable by the end of the experiment.

Rats were assigned to an overall active or control group, depending on whether they had raised sustained anti-cotinine antibody titres or not: Rats immunised entirely with "new" TT-CotSH that had raised no antibodies, and those only given one initial "old" TT-CotSH injection, that had hardly raised any antibodies, were combined to form the mean control group, while all others were assigned to the mean active group. Overall, the maximum titres measured at the end of this Trial were extremely low ($1:492 \pm 70$) compared to previous Trials (see Figure 2.9 for titre comparison across trials).

Figure 2.7: Immune response to anti-cotinine vaccination (Trial 4)

Rats were immunised with 5 μ g TT-CotSH in AS02v adjuvant on the days indicated (arrows). Blood samples were collected at each timepoint and serum mid-point titres of all vaccinated rats ($n = 18$) were determined by ELISA, using trans-4-thiol cotinine coated plates.

Antibody titres are expressed as the dilution producing 50% maximal optical density on ELISA (i.e. mid-point titres). Data presented are means \pm S.E.M.

“Old” refers to conjugates initially used at the time the first rats entered the vaccination phase; once the “new” (i.e. fresh batch of) conjugates arrived, these were used for subsequent injections. For detailed background on groups and vaccination problems, see section 2.3.3.3 (under “Trial 4”).

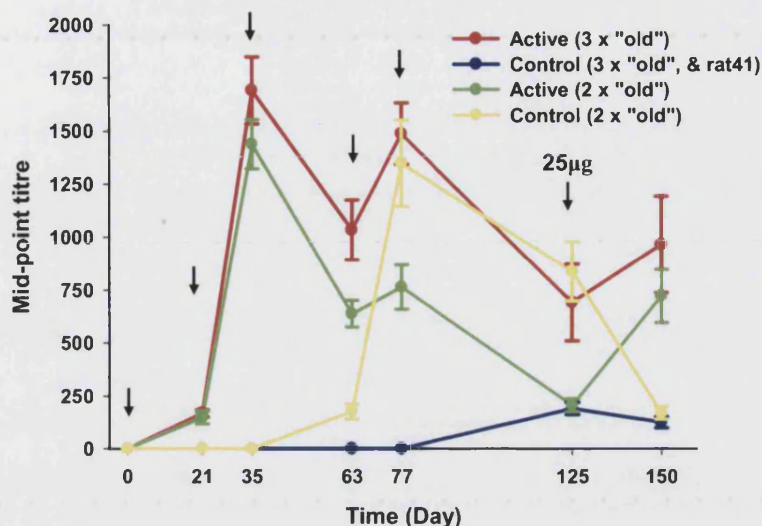
Brightly coloured symbols give titres of rats grouped by their original intended category (active / control) as well as the number of injections of “old” / “new” conjugates they received. The darker symbols give means of those animals assigned to control / active after determination of anti-CotSH titres.

Trial 5

All 48 rats were initially vaccinated twice with conjugates produced from the original ("old") batch of cotinine derivative (CotSH). Once the conjugates derived from the fresh batch of CotSH arrived from GSK Rixensart ("new" conjugates), these were used for vaccinations. Rats were immunised over 3 days in groups of 16. Due to the timing of the arrival of the new conjugates, the first 16 rats received a third injection of "old" conjugates; whereas the remaining rats were given "new" conjugates for their third vaccination. For all rats, the 2 subsequent booster injections consisted of "new" conjugate.

Anti-cotinine titres were initially determined for "active" and "control" vaccinated groups. It was not until the determination of antibody levels in samples taken on day 77 (which took place while the locomotor experiments (see section 4.2.2.6) were underway) that suspicions were confirmed that the vials containing the new conjugates might have been labelled incorrectly. Splitting the "active" and "control" vaccinated groups into those that had received 3 doses (1-16) and those that had received only 2 injections of "old" conjugates (remaining rats), provided further evidence (see Figure 2.8): in "control" (TT-Cysteine immunised) animals among rats 1-16, titres were still non-existent at day 77, whereas the remaining "control" rats had developed anti-cotinine titres similar to the rats in the "active" group (among rats 1-16). On the other hand, in the group of "active" (TT-CotSH immunised) rats that had been switched to the "new" conjugate after only 2 injections, anti-cotinine titres decreased more rapidly than in "active" rats among subjects 1-16, that had received 3 vaccinations with "old" conjugates.

Rat 41 provided further confirmation: this "control" rat had been erroneously immunised with "new" TT-CotSH conjugate on one occasion. However, the level of anti-cotinine antibodies for this rat was lower, not higher, than those found in the other rats belonging to the "control" group vaccinated with "new" conjugates on 3 occasions, and very similar to the titres of the "control" rats among individuals 1-16. Titres for rat 41 were therefore included in the results for the latter group of rats (see Figure 2.8).

Figure 2.8: Immune response to anti-cotinine vaccination (Trial 5)

Rats were immunised with 5 µg TT-CotSH in AS02v adjuvant on the days indicated (arrows). Blood samples were collected at each timepoint and serum mid-point titres of vaccinated rats were determined by ELISA, using trans-4-thiol cotinine coated plates.

Antibody titres are expressed as the dilution producing 50% maximal optical density on ELISA (i.e. mid-point titres). Data presented are means \pm S.E.M.

For detailed background on groups and vaccination problems, see section 2.3.3.3 (under "Trial 5"). In brief: conjugates used for initial vaccinations ("old") were replaced by a fresh batch after 2-3 injections (depending on animals). Rats are grouped according to the number of injections of "old" conjugates received before the switch took place. Rat41 was erroneously injected with "new TT-CotSH" instead of "new TT-Cysteine", developing lower, instead of higher, antibody titres than comparable animals as a result.

Animals received a final vaccination of 25 µg "old" conjugates in an attempt to restore original active / control groups.

On day 125, all rats were given an additional booster, consisting of 25 µg of "old" conjugates plus AS02v, in an attempt to increase the levels of anti-cotinine antibodies in "active" rats and reduce any further increases of anti-cotinine titres in "control" rats. Although this did help to restore the original "active" and "control" groups to a certain extent (see Figure 2.8), the increase in titres in the "active" rats was not as large as anticipated, resulting in very low final anti-cotinine antibody titres ($1:806 \pm 114$) compared to previous trials (see Figure 2.9 for titre comparison across trials). For the withdrawal experiments (see Chapter 4), the original designation of rats to active and control groups was

maintained (with the exception of rat 41; see above), however rats from within these groups were selected on the basis of titres raised, i.e. only those with the highest anti-cotinine antibody titres were used for active groups, and those with the lowest titres for controls.

Figure 2.9: Comparison of anti-cotinine antibody titres across trials

| Trial | Mean \pm SEM | Range |
|--------------|----------------------------------|------------------|
| 1 | 1:7061 \pm 1973 | 1:1951 – 1:13709 |
| 2 | 1:9832 \pm 826 | 1:3258 – 1:27315 |
| 3 | 1:3008 \pm 413 | 1:635 – 1:9428 |
| 4 | 1:397 \pm 61 | 1:213 – 1:621 |
| 5 | 1:806 \pm 114 | 1:158 – 1:2234 |

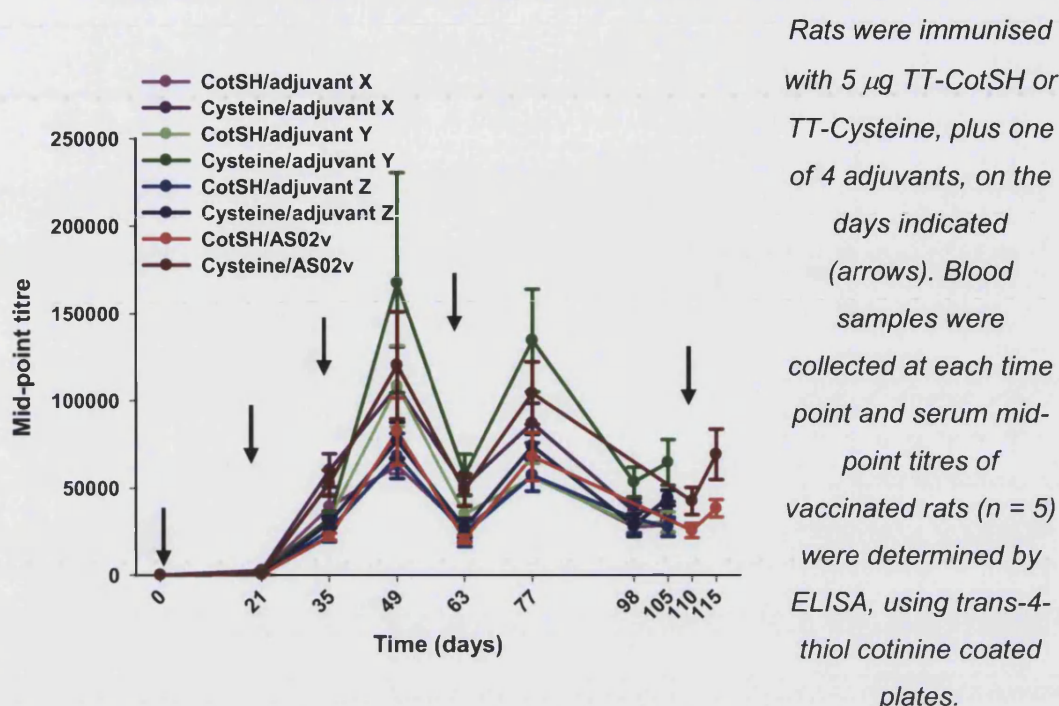
Titres shown are the final titres for animals immunised with TT-CotSH, or, in the case of Trials 4 & 5, those animals designated as belonging to the “active” group.

2.3.3.4 Anti-TT titres

No anti-TT antibodies were detected in any of the rats prior to the start of vaccinations (in any of the trials). As for antibodies against cotinine, mid-point titres of antibodies against TT increased from one vaccination to another, and also required regular booster injections for maintenance of titres. Levels of anti-TT antibodies were generally 10-fold greater than those of anti-cotinine antibodies (see Figure 2.9 – 2.11).

Trial 1 (Adjuvant comparison)

In Trial 1, anti-TT antibody levels increased in all adjuvant groups as a result of repeated vaccinations (see Figure 2.10) in a similar pattern to the anti-cotinine antibody titres (see section 2.3.3.3); however anti-TT titres were much higher. As with the anti-cotinine antibodies, levels dropped off over time and increased again after the booster injection on day 63. A comparison between groups on day 77 revealed a significant effect of conjugate ($F(\text{conjugate})_{1,32} = 14.625$, $p=0.0006$), with titres in groups vaccinated with control vaccine (TT-Cysteine) generally higher (~1:80 000 – 1:130 000) than those in rats vaccinated against cotinine (~1:60 000 – 1:70 000; data not shown).

Figure 2.10: Immune response to TT component of vaccines (Trial 1)

Antibody titres are expressed as the dilution producing 50% maximal optical density on ELISA (i.e. mid-point titres). Data presented are means \pm S.E.M.

In the groups vaccinated with AS02v adjuvant, an increase in anti-TT antibody levels was observed after their second booster injection on day 110 (see Figure 2.10); however it was not as marked as that observed for the anti-cotinine antibodies (see Figure 2.4). Final titres for the AS02v adjuvant groups were \sim 1:40 000 for rats immunised with TT-CotSH and \sim 1:70 000 for the controls (immunised with TT-Cysteine).

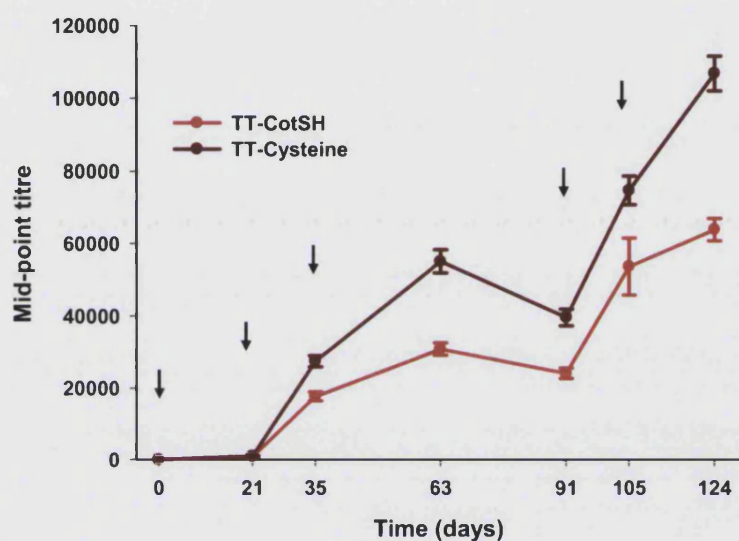
Trial 2

Anti-TT antibody levels increased from one vaccination to the next (see Figure 2.11) and were generally higher in the control group compared to the rats receiving active vaccine.

Titres of \sim 1:55 000 (TT-CotSH) and \sim 1:75 000 (TT-Cysteine) were reached by day 105, prior to locomotor experiments. There was a significant difference in final titres between control and active groups ($p < 0.0001$) at the end of the behavioural experiments, and mean titres had increased further to \sim 1:60 000 (TT-CotSH) and \sim 1:110 000 (TT-Cysteine), which was somewhat higher than in

Trial 1 (see above). There were no further significant differences in anti-TT antibody levels post-locomotor between treatment groups, i.e. no effects of treatment or pairing (data not shown).

Figure 2.11: Immune response to TT component of vaccines (Trial 2)

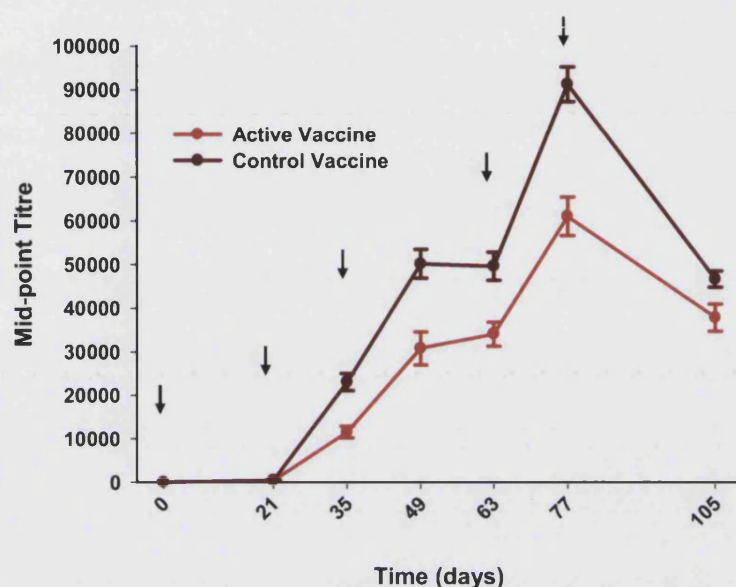


Rats were immunised with 5 μ g TT-CotSH or TT-Cysteine in AS02v adjuvant on the days indicated (arrows). Blood samples were collected at each timepoint and serum mid-point titres of vaccinated rats ($n = 48$) were determined by ELISA, using trans-4-thiol cotinine coated plates.

Antibody titres are expressed as the dilution producing 50% maximal optical density on ELISA (i.e. mid-point titres). Data presented are means \pm S.E.M.

Trial 3

Anti-TT antibody titres for Trial 3 are shown in Figure 2.12. Again, anti-TT antibody levels increased steadily as a result of repeated vaccinations (see Figure 2.12), however the final booster injection failed to produce a further increase, with titres decreasing from day 77 to sacrifice (day 103-106). Final mean titres for control animals were significantly greater than those receiving active vaccine ($\sim 1:40\,000$ (TT-CotSH) and $\sim 1:45\,000$ (TT-Cysteine); $p = 0.0174$).

Figure 2.12: Immune response to TT component of vaccines (Trial 3)

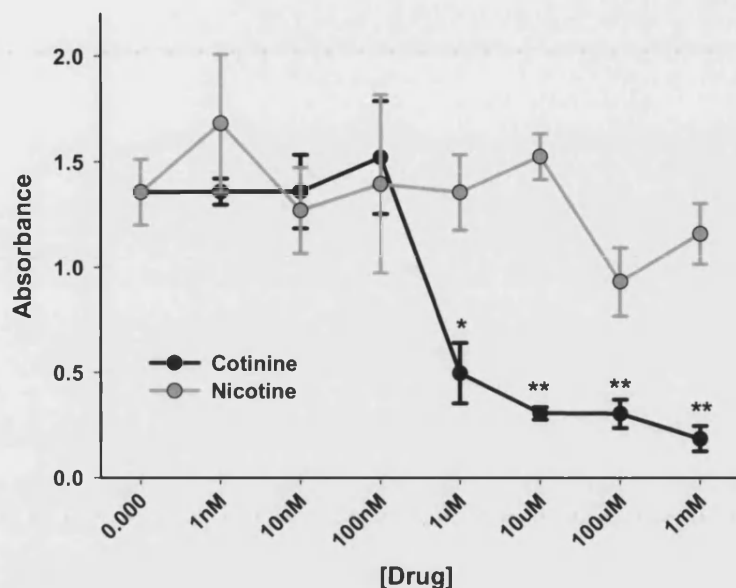
Rats were immunised with 5 μ g TT-CotSH or TT-Cysteine in AS02v adjuvant on the days indicated (arrows). Blood samples were collected at each timepoint and serum mid-point titres of vaccinated rats ($n = 24$) were determined by ELISA, using trans-4-thiol cotinine coated plates.

Antibody titres are expressed as the dilution producing 50% maximal optical density on ELISA (i.e. mid-point titres). Data presented are means \pm S.E.M.

2.3.3.5 Competitive ELISA – specificity of anti-CotSH antibodies

The specificity of anti-CotSH antibodies for cotinine versus nicotine was examined by means of a competitive ELISA.

Antibodies showed much greater specificity for cotinine than nicotine (see Figure 2.13), in agreement with previous findings (Pashmi 2004). Cotinine was able to compete with the CotSH plate coating for antibody binding causing a significant reduction from a concentration of 1 μ M upwards (compared to pre-incubation with buffer alone), whereas competition by nicotine was not significant, with only a trend starting to occur from around 100 μ M upwards.

Figure 2.13: Specificity of anti-CotSH antibodies for cotinine

Sera taken on day 77 from rats immunised with 5 μ g TT-CotSH ($n = 4$ replicates of pooled sera; Trial 3) were assayed in a competitive ELISA, using plates coated with 0.05 μ g/ml trans-4-thiol cotinine. Sera were pre-incubated with nicotine or cotinine (1 nM – 1 mM) overnight at 4 °C prior to ELISA.

Data presented are means \pm S.E.M.; * $p < 0.05$, ** $p < 0.01$ (compared to baseline)

2.3.4 Discussion

The main characteristics of a vaccine that are relevant to the treatment of nicotine addiction are its immunogenicity (i.e. the ability to raise antibodies in response to vaccination), and the affinity and specificity of the antibodies produced. Specificity refers to the ability of antibodies to recognise immunogen (in this case cotinine) over other metabolites, whereas affinity describes the strength with which the target is bound by the antibody.

The ability of TT-CotSH to elicit the production of anti-cotinine antibodies in response to vaccination is key to the efficacy of the anti-cotinine vaccine. Previous studies have focussed on optimising the cotinine derivative used, as well as the carrier protein to which it is conjugated, with a much greater immune response induced after coupling to tetanus toxoid (TT) compared to bovine serum albumin (BSA; Pashmi 2004). Anti-nicotine conjugate vaccines have utilised keyhole limpet hemocyanin (KLH; Hieda et al 1997, de Villiers et al 2002, Carrera et al 2004), recombinant cholera toxin B subunit (Cerny et al 2002) and recombinant pseudomonas exoprotein A (Pentel et al 2000), among others.

In order to maximise the antibody production as a result of vaccination, we compared several adjuvants/adjuvant combinations for their ability to enhance the immune response to the vaccine (see Chapter 1), including AS02v, which Pashmi had found to produce a greater immune response than alum alone as adjuvant (Pashmi 2004). As we observed no huge differences in terms of mean anti-cotinine antibody titre between groups, and the group receiving AS02v had the highest mean antibody levels (see Figure 2.4), AS02v was used as adjuvant in all subsequent experiments. Studies on anti-nicotine vaccines, on the other hand, have used Freund's adjuvant in preclinical studies. This is a relatively aggressive adjuvant (and therefore not licensed for use in humans), which is able to elicit a substantial immune response. This explains why antibody titres achieved in these studies are consistently greater than 1:10 000 (Hieda et al 1997, de Villiers et al 2004, Satoskar et al 2003).

The second booster vaccination significantly increased anti-cotinine antibody levels in the first trial (see Figure 2.3), so this additional injection was included in the vaccination schedules thereafter (see section 2.3.2.3). The anti-cotinine titres achieved in Trial 2 (see Figure 2.5) were the highest, compared with the other trials (see Figure 2.9), which may have been the result of the longer interval between the third vaccination and the first booster injection, giving the immune response more time to develop. Unfortunately we were unable to repeat this time schedule in the other trials, due to time constraints on equipment usage.

It is unclear why titres achieved in Trial 3 (see Figure 2.6) were only half as great as those obtained in Trial 1, although a possible cause might be the shorter gap between the two booster injections in Trial 3. The other possibility is that the derivative (CotSH), from which the active conjugate was derived, might have started to degrade. We therefore synthesised a fresh batch of CotSH and had new conjugates made (by GSK Biologicals in Belgium). Due to the mislabelling of these new conjugates (i.e. TT-CotSH labelled as TT-Cysteine and vice versa), anti-cotinine antibody titres obtained in Trials 4 & 5 were very low. But even in those rats that had received only "new" conjugates (Trial 4; see Figure 2.7), titres did not reach levels comparable to previous trials. This might have been the result of reduced conjugation of the new derivative to TT, possibly due to slight differences in chemistry between the two batches of

derivative as they were produced by different laboratories. To complicate matters further, rats used for Trials 4 & 5 were of the Lister hooded strain, whereas the preceding three trials had used Sprague-Dawleys. In addition, rats in Trial 4 were also self-administering nicotine during the vaccination phase, which might have had an impact on the magnitude of the immune response as chronic nicotine administration has been shown to suppress immune responses in rats (Kalra 2002).

In order to get a better idea of the overall magnitude of the immune response elicited by vaccination with TT-CotSH and evaluate the immune response to the control vaccine (TT-Cysteine), the levels of antibodies raised against the TT portion of the conjugates were also examined. These were found to be consistently much higher than those raised against cotinine, an indication of the much greater immunogenicity of TT compared to cotinine. However, the anti-TT antibody levels observed in control-vaccinated rats were also much higher than those in rats injected with active vaccine. This might, at least in part, be a result of some of the anti-TT antibodies from rats vaccinated with TT-Cysteine recognising the TT-Cysteine used to coat the ELISA plates for this assay slightly better than anti-TT antibodies raised by rats injected with TT-CotSH. Antibodies recognise a very specific epitope on the antigen, and some of the anti-TT antibodies might have been raised against a part of the linking portion of the conjugate, connecting the cysteine molecules to the TT carrier protein. Such a sequence would be much less likely to be recognised by an anti-TT antibody raised against the linking portion between CotSH and TT.

Generally, the anti-cotinine antibody titres obtained in response to vaccination with TT-CotSH were disappointing. Only Trial 2 came close to the antibody levels achieved by vaccination against nicotine (Hieda et al 1997, de Villiers et al 2004, Satoskar et al 2003). This might be at least partially due to the larger dose of conjugate (25 – 100 µg) administered in studies examining anti-nicotine vaccines (Hieda et al 1997, Lindblom et al 2002, Satoskar et al 2003, LeSage et al 2006). Previous work (Pashmi 2004) on the anti-cotinine vaccine involved the comparison of anti-cotinine antibody titres across several doses of TT-CotSH, with the conclusion that 5 µg was the optimum dose (over 1 and 25 µg). However, upon closer examination the titres obtained in that study were very

variable, ranging from ~1:4000 to ~1:54000 in the 5 µg group, with a mean titre of ~1:24000.

The specificity of antibodies produced can have a bearing on the safety profile of a vaccine, with greater specificity reducing the risk of competition for binding by other compounds, leading to improvements in safety and a reduced risk of side effects. The anti-cotinine antibodies raised as a result of vaccination with TT-CotSH were shown to be specific for cotinine with only a tendency towards cross-reactivity for nicotine, which was not significant even at the highest nicotine doses examined. This confirms previous findings by Pashmi (2004), who observed that anti-cotinine antibodies were specific for cotinine with little or no cross-reactivity for any of the metabolites studied, although a small degree of competition by norcotinine was detected. However, while norcotinine has been shown to be present and metabolised in rat brain, there is no literature to suggest that it has any pharmacological activity (Crooks & Dwoskin 1997, Ghosheh et al 2001).

2.4 Chapter conclusions

We were able to establish the methodology for generating an anti-cotinine vaccine, as the first step in evaluating a novel approach to combating tobacco smoking. The rationale for this approach is that this primary metabolite of nicotine competes with nicotine, reducing its effect, and therefore causing smokers to increase their intake of nicotine. The presence of cotinine would also compromise the efficacy of nicotine replacement treatments. The weak pharmacological activity of cotinine was verified in *in vitro* assays of dopamine release from striatal slices. As an agonist, cotinine is approximately 1000 times less potent than nicotine, in agreement with previous reports (Anderson et al 2000). This is also reflected in its weak interaction with nicotinic receptor binding sites (Ghosheh et al 2001, Dwoskin et al 1999).

Pashmi (2004) had previously shown that brief exposure of striatal slices to a high concentration of cotinine significantly decreased responses to a subsequent stimulation with nicotine. This is compatible with receptor desensitisation by cotinine. Given that the average smoker sustains high plasma cotinine concentrations (~15 times higher than peak concentrations of

nicotine; Hatsukami et al 1998), this ability to “antagonise” nicotine’s actions is likely to influence smoking patterns. Using immunisation to raise antibodies that bind cotinine, thus preventing it from entering the brain, should combat this antagonism, thereby rendering nicotine replacement therapy more effective.

Both nicotine and cotinine are too small to be recognised by the immune system under normal circumstances. In order to elicit an immune response they need to be linked to an immunogenic carrier protein, resulting in a conjugate vaccine. Several anti-nicotine vaccines have been created by conjugating nicotine or a structurally related compound (i.e. hapten) to a carrier protein (Hieda et al 1997, de Villiers et al 2002, Carrera et al 2004, Cerny et al 2002, Pentel et al 2000). In the present study we chose TT as the carrier, because of its use in human vaccines. Coupling cotinine to TT required introduction of a linker arm bearing a reactive group. Introduction of a thiol group attached to an amide spacer via the C4 position of cotinine facilitated the efficient coupling to TT, without compromising the antigenic recognition of cotinine, as endorsed by the specificity of the anti-cotinine antibodies.

Repeated immunisation of rats with TT-CotSH resulted in the production of anti-cotinine antibodies and titres increased with each booster. Preclinical studies of anti-nicotine vaccines have reported higher titres (Hieda et al 1997, de Villiers et al 2004, Satoskar et al 2003) but this is likely to reflect differences in the adjuvants used: most of the protocols for studies of anti-nicotine vaccines have used the more immunogenic Freund’s adjuvant, whereas we used an adjuvant developed with a view towards future use in humans, to give a more realistic picture of the magnitude of the immune response which might be achieved in patients.

In order to maximise the therapeutic effect and minimise potential side effects of an anti-cotinine vaccine, antibodies raised against cotinine need to be specific. Antibodies raised in response to immunisation with TT-CotSH recognised cotinine with very little cross-reactivity for other nicotine metabolites or nicotine itself, and no antibodies were detected in any control animals, demonstrating that the structure of the conjugate vaccine permits recognition of the cotinine-specific component by the immune system. Studies on anti-nicotine vaccines

have observed similar specificity (Hieda et al 1997, de Villiers et al 2002, Cerny et al 2002, Maurer et al 2005).

Having characterised the immune response induced by vaccination with TT-CotSH, the effects of anti-cotinine antibodies on a variety of nicotine-induced responses were examined both in vitro and in vivo.

Chapter 3

**Effects of immunisation
on nicotine / cotinine
distribution and nicotine
binding sites**

Chapter 3

Effects of immunisation on nicotine / cotinine distribution and nicotine binding sites

3.1 Chapter introduction

As discussed in Chapter 2, immunisation of rats with TT-CotSH elicited an immune response, which resulted in the production of anti-cotinine antibodies. These antibodies are specific for cotinine and do not recognise nicotine or other nicotine metabolites.

According to the hypothesis set out in the Chapter 1 (see section 1.9), such anti-cotinine antibodies should bind cotinine, retaining it in the bloodstream and preventing it from crossing the blood-brain-barrier. In order to see whether this was the case, we studied the distribution of nicotine and cotinine, in both the blood and the brain, after different schedules of nicotine administration (see section 3.2).

Preventing cotinine from entering the brain should also remove or at least reduce any cotinine-mediated antagonism of nicotine's actions. Nicotine administration is known to upregulate nicotine binding sites. We therefore examined the effect of vaccination on [^3H]epibatidine and [^{125}I] α Bungarotoxin binding levels after administration of nicotine by daily injection or chronic subcutaneous infusion to see whether antagonism of cotinine's actions had any effect on nicotine-induced nAChR upregulation (see section 3.3).

3.2 Nicotine & cotinine distribution in blood and brain

3.2.1 Introduction

Nicotine is absorbed rapidly from cigarette smoke, enters the arterial circulation and is quickly distributed to body tissues. Nicotine levels then start to fall, as a result of uptake by peripheral tissues and elimination from the body. It takes just 10-20 s for nicotine to pass through the brain (Benowitz 1996). This rapid delivery of nicotine results in a relatively intense pharmacological response, due to high arterial levels entering the brain and effects occurring rapidly, before there is adequate time for the development of tolerance. While tolerance to the

effects of nicotine takes days or even weeks to develop and can persist for a similar length of time after discontinuation of nicotine administration, desensitisation of nAChRs can occur within seconds of smoking a cigarette. However, nicotine levels in the brain decline between cigarettes, providing an opportunity for resensitisation of receptors so that positive reinforcement can occur to some extent with successive cigarettes (Dani & Heinemann 1996). In contrast to cigarette smoking, slow release of nicotine, as seen with transdermal NRT, is much less reinforcing, since blood nicotine levels rise gradually, allowing tolerance to develop (Benowitz 1996). Peak arterial levels are also much lower with transdermal nicotine compared to cigarette smoking (see section 1.6.1.1), even with a similar daily nicotine intake. The lack of quick relief from withdrawal symptoms as a result of the slow nicotine delivery is one of the major drawbacks of transdermal NRT, and one of the key reasons why so many quitters are unable to remain abstinent.

Since the elimination half-life of nicotine is approximately 2-3 hours, nicotine levels can accumulate over 6-8 hours during regular smoking or nicotine dosing (Benowitz 1996). Nicotine is rapidly metabolised to cotinine by cytochrome P450 enzymes (see section 1.2). Cotinine, the major metabolite of nicotine, has a half-life of 15-19 hours in humans and therefore accumulates in the body to a much greater extent than nicotine (Herzig et al 1998).

Immunisation against nicotine or administration of anti-nicotine antibodies has been shown to significantly increase blood nicotine levels and reduce distribution of nicotine to the brain (Hieda et al 1997 & 1999, Keyler et al 1999, Pentel et al 1999, Satoskar et al 2003, de Villiers et al 2004). Maternal vaccination has also been observed to reduce foetal exposure to nicotine (Keyler et al 2003 & 2005). These effects appear to be due to an increase in levels of bound nicotine in the blood, as brain nicotine concentration was found to be inversely related to both serum nicotine concentration and nicotine protein binding in serum, indicating that nicotine was being sequestered by anti-nicotine antibodies in the vaccinated animals (Hieda et al 1999). Antagonism of nicotine in this manner might reduce the rewarding effects of the drug, but it would also significantly reduce the ability of NRT to alleviate the withdrawal symptoms usually associated with smoking cessation.

In order for the anti-cotinine vaccine to improve the efficacy of NRT in smoking cessation, the antibodies raised as a result of immunisation would need to bind and retain cotinine in the bloodstream, preventing it from crossing the blood-brain-barrier and antagonising nicotine's actions. To see if this was the case, we examined the distribution of both nicotine and cotinine in blood and brain following different nicotine administration schedules.

3.2.2 Methods

3.2.2.1 Nicotine regimes

To study the effect of intermittent nicotine exposure, we used rats that had been given daily nicotine (0.5 mg/kg) or saline injections for 15 days (see section 4.2.2.5) as part of locomotor experiments. Animals were culled 5 min after their last injection.

We also examined the effects of chronic nicotine exposure. Rats used for these experiments had received a chronic infusion of nicotine (3.16 mg/kg/day) for 7 days via osmotic minipumps (OMP) as part of the mecamylamine-precipitated nicotine withdrawal experiments (see section 4.5.2.3). Control rats were implanted with a "blank". After the behavioural experiments, blood samples were taken and rats were perfused transcardially with PBS (see section 3.2.2.3) prior to dissecting out the brain.

3.2.2.2 Preparation of brain tissue for analysis of nicotine & cotinine levels

3.2.2.2.1 Unperfused brain

Rats were killed by cervical dislocation and the brain dissected on ice. One hemisphere was added to a tube containing 1 ml of 0.1 M HCl. After determining the weight of the tissue, 1:2 homogenates of brain:HCl were prepared. These were stored at -20°C and shipped to ABS Laboratories for analysis (see section 3.2.2.3). The other hemisphere was quickly frozen in liquid nitrogen and used for radioligand binding experiments (see section 3.3).

3.2.2.2.2 Perfusion

After completing the mecamylamine-precipitated withdrawal experiments (see section 4.5), rats were given a lethal dose of sodium pentobarbital (1 ml/kg) by intraperitoneal injection. Once the footpad reflex was no longer present, the ribcage was exposed by cutting away the fur and skin. A small incision was made to locate the xyphoid cartilage (sternum), and, holding the cartilage firmly, the ribcage was opened up towards the head, exposing the heart. A cannula was inserted into the left ventricle and clamped in place. A small incision was made in the right atrium to create an outlet for blood and perfusion fluids, and the pump turned on immediately to allow perfusion to commence. Each rat was perfused transcardially with ~100 ml chilled PBS (0.1 M, pH 7.4).

Once perfusion was complete, the pump was switched off and the cannula removed. The brain was dissected out as quickly as possible, and the brain tissue prepared as described above (see section 3.2.2.2.1). The second hemisphere was again quickly frozen in liquid nitrogen for use in radioligand binding experiments (see section 3.3).

3.2.2.3 Analysis of blood nicotine & cotinine levels

Blood samples taken when the animals were culled were treated as described in section 2.2.4 to obtain sera. The samples were shipped to ABS Laboratories, London, for determination of nicotine and cotinine concentration by gas chromatography.

3.2.2.4 Data Analysis

Data shown are means \pm S.E.M. for each group. Unpaired t-tests were used to examine significant differences between treatment groups.

3.2.3 Results

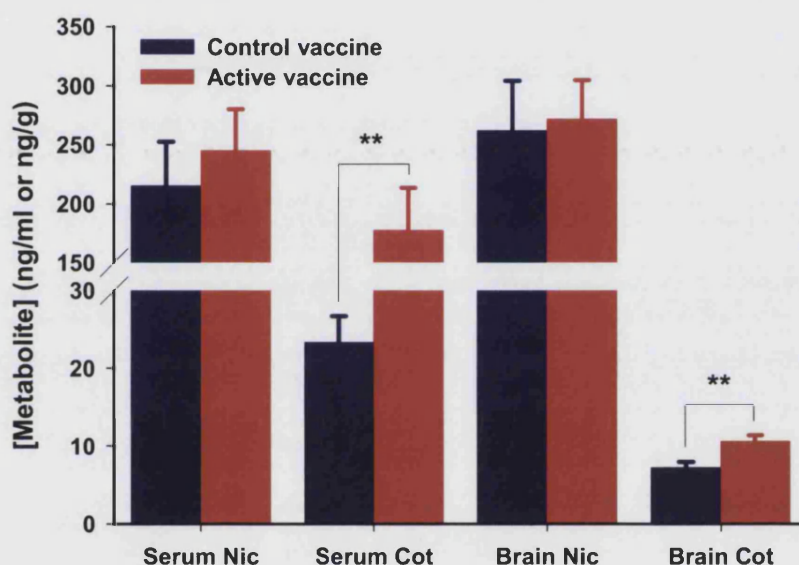
3.2.3.1 Nicotine / cotinine distribution following once-daily injections of nicotine for 15 days

All saline-treated rats consistently had nicotine and cotinine levels (blood & brain) of ≤ 1 ng/ml (data not shown).

Cotinine levels were markedly lower than nicotine levels in all nicotine-treated groups. This was due to the short interval (5 min) between administering the

nicotine and obtaining the samples, during which only a fraction of the dose was converted to cotinine. Rats that had been immunised with control vaccine (TT-Cysteine) and had been administered nicotine for 15 days had mean serum nicotine and cotinine levels of 215 ± 37 and 23 ± 3 ng/ml, respectively (see Figure 3.1). Their mean brain nicotine concentration was 262 ± 42 ng/g, with brain cotinine levels again much lower (7 ± 1 ng/g).

Figure 3.1: Nicotine & cotinine distribution in blood and brain after 15-day intermittent nicotine administration



Rats that had been immunised with either active (TT-CotSH) or control (TT-Cysteine) vaccine were given daily nicotine (0.5 mg/kg) or saline injections for 15 days. Animals were culled 5 min after their last injection. Blood samples were taken and treated as described in section 2.2.4 to obtain sera, and nicotine & cotinine concentration determined by gas chromatography.

Data shown are means \pm S.E.M ($n = 6$). ** $p < 0.01$

Nicotine levels in both serum (245 ± 35 ng/ml) and brain (272 ± 33 ng/g) of rats immunised against cotinine did not differ from those receiving control vaccine (see Figure 3.1). On the other hand, in rats immunised against cotinine, serum cotinine levels were significantly higher (177 ± 36 ng/ml; $p = 0.0017$) compared to control, indicating that cotinine was being retained in the bloodstream by the anti-cotinine antibodies. Brain cotinine levels were also significantly elevated (11 ± 1 ng/g; $p = 0.0098$) in rats receiving active vaccine (TT-CotSH) compared

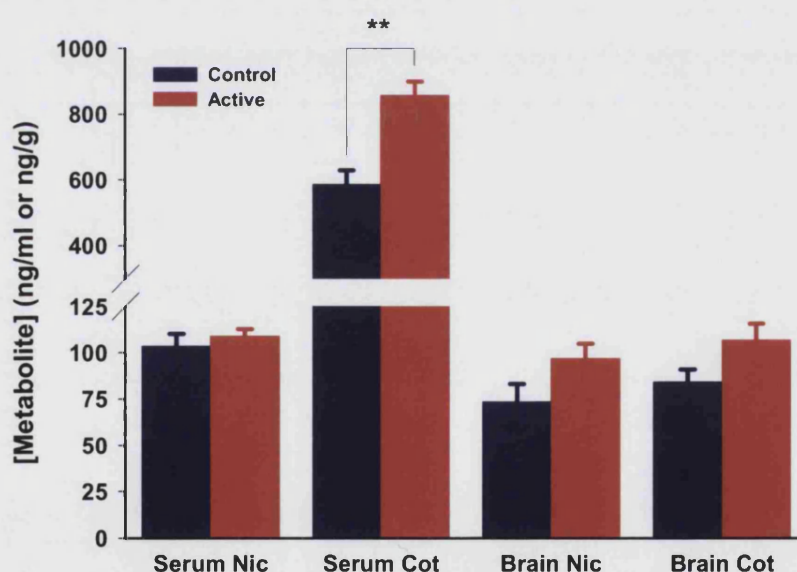
to controls; however the increase in cotinine concentrations observed in serum (670 %) was much greater than in brain (57 %).

Because these results could reflect the contamination of brain tissue with blood occurred during dissection, animals were perfused with PBS prior to dissecting the brain in subsequent experiments (see section 3.2.2.2.2), in order to remove as much blood as possible from the brain itself and any surrounding tissue containing blood vessels likely to be damaged during dissection.

3.2.3.2 Nicotine / cotinine distribution following 7-day chronic nicotine infusion

Saline-treated rats consistently had nicotine and cotinine levels (blood & brain) of ≤ 1 ng/ml (data not shown).

Fig 3.2: Nicotine & cotinine distribution in blood and brain after 7-day chronic nicotine infusion



Rats received a chronic infusion of nicotine (3.16 mg/kg/day) for 7 days via osmotic minipumps (OMP). Control rats were implanted with a "blank". On day 7, blood samples were taken and rats were perfused transcardially with PBS (see 3.2.2.2.2) prior to removing the brain.

Blood samples were taken and treated as described in section 2.2.4 to obtain sera, and nicotine & cotinine concentration determined by gas chromatography.

Data shown are means \pm S.E.M ($n = 6-8$). ** $p < 0.01$

Control immunised animals had mean serum nicotine and cotinine levels of 104 ± 7 and 586 ± 43 ng/ml, respectively (see Figure 3.2), and brain concentrations of 73 ± 10 ng/g (nicotine) and 84 ± 7 ng/g (cotinine).

Nicotine levels (serum and brain) in the active (TT-CotSH) vaccine groups were not significantly different compared to controls (109 ± 4 ng/ml and 97 ± 8 ng/g, respectively). Cotinine levels in the brain were also not significantly different (107 ± 9 ng/g; $p=0.0725$) from controls, although a small trend towards higher levels was observed with immunisation against cotinine. This supported the suspicion that in the previous experiment the difference in brain cotinine levels had been due to contamination of the brain samples with blood (see section 3.2.3.1). As had been the case in the previous experiment, serum cotinine levels (857 ± 41 ng/ml; $p = 0.0011$) were significantly higher in rats immunised against cotinine compared to those given control vaccine (TT-Cysteine).

3.2.4 Discussion

Nicotine and cotinine distribution in control groups

In our first study, serum cotinine levels measured were relatively low compared to nicotine levels, most likely because the samples were taken quite soon (5 min) after nicotine administration, before much of the dose had been metabolised to cotinine. In the second study, after chronic infusion of nicotine for a week, the plasma cotinine concentration was much greater, due to the gradual accumulation of cotinine over time.

In a moderate smoker, plasma nicotine levels average 10-50 ng/ml, whereas cotinine levels can reach up to 250-350 ng/ml (Lockman et al 2005). In rats, chronic infusion of nicotine (4.5 mg/kg/day) for 28 days produces comparable plasma levels (72 and 469 ng/ml for nicotine and cotinine, respectively; Lockman et al 2005). These values are not that dissimilar from those observed in our second (chronic) study, although the dose we used was slightly lower, and the animals only received nicotine for 7 days.

Nicotine and its metabolites have very different half-lives: Ghosheh et al determined that in rats, brain half-lives of nicotine, cotinine and nornicotine were 52, 333, and 166 min, respectively (Ghosheh et al 1999). Due to their different elimination rates, the relative balance of these metabolites in the body also varies depending on the time point, at which a measurement is taken, as well as

the route and frequency of administration. Chronic infusion of 2.4 mg/kg/day for 10 days resulted in plasma and brain nicotine levels of 43 ng/ml and 120 ng/g, respectively (Rowell & Li 1997). The same dose given in the form of multiple injections on the other hand produced very different plasma & brain concentrations: 140 ng/ml & 520 ng/g (8 x 0.3 mg/kg), 202 ng/ml & 688 ng/g (4 x 0.6 mg/kg), or 535 ng/ml & 1133 ng/g (2 x 1.2 mg/kg). Brain nicotine levels tended to be several times higher than plasma levels in all injection regimes examined (Rowell & Li 1997), whereas we observed quite similar nicotine concentrations in serum and brain in both of our studies. However, we observed a tendency towards lower nicotine and higher cotinine levels (blood & brain) after chronic nicotine administration (see Figure 3.2), compared to the repeated injection regime (see Figure 3.1).

Four hours after a single s.c. injection of 0.54 mg/kg nicotine, Crooks & Dwoskin observed brain concentrations of nicotine and cotinine in the rat of 12 and 45 ng/g, respectively (Crooks & Dwoskin 1997). The brain nicotine levels we found after injection of 0.5 mg/kg are much higher than that (~250 ng/g), and the cotinine levels much lower (~10 ng/g); however, our samples were collected just 5 min after nicotine administration. As nicotine has such a short half-life in the rat (<1 h), nicotine levels could conceivably fall to a level comparable to that detected by Crooks & Dwoskin in the space of 4 hours, while cotinine levels could rise accordingly as more nicotine is metabolised.

Effects of vaccination on nicotine & cotinine distribution

In Chapter 2 we showed that the antibodies raised in response to immunisation against cotinine are specific for cotinine and do not recognise nicotine or other metabolites. This is supported by the observation that there was no significant difference between vaccinated and control rats in terms of nicotine levels in serum or brain after intermittent nicotine administration or chronic infusion (see Figures 3.1 & 3.2).

Plasma cotinine levels, on the other hand, were significantly increased (7.7-fold after repeated injections and 1.5-fold following chronic administration) in those rats immunised against cotinine. This is consistent with the hypothesis that antibodies raised in response to anti-cotinine vaccination bind cotinine,

preventing it from entering the brain, and retain it in the blood (still bound to the antibody).

Results have confirmed a similar hypothesis for anti-nicotine vaccines: Total and bound serum nicotine concentrations appear to be significantly higher in vaccinated rats compared to controls after administration of a bolus infusion of nicotine (Hieda et al 1997, Keyler et al 1999, de Villiers et al 2004), while unbound serum nicotine is lower (Satoskar et al 2003), resulting in a reduced clearance rate. Distribution of nicotine to the brain is also significantly reduced in rats vaccinated against nicotine (Keyler et al 1999, Hieda et al 1999, Pentel et al 1999, Satoskar et al 2003, de Villiers et al 2004). Chronically infused nicotine showed a similar pattern of distribution, but differences were generally smaller than after a single nicotine dose. Vaccine efficacy in reducing nicotine distribution to the brain does not appear to be compromised by concurrent nicotine administration (Hieda et al 2000).

Passive immunisation with anti-nicotine antibodies prior to nicotine administration also reduced brain nicotine levels in a dose-related manner (65% reduction at the highest IgG dose) and increased serum nicotine dose-dependently (Pentel et al 1999). No differences in serum cotinine levels were observed as a result of either active or passive immunisation against nicotine (Pentel et al 1999, de Villiers et al 2004).

Furthermore, active or passive maternal immunisation reduced nicotine distribution to both maternal brain (44-47%) and foetal brain (17-39%) after a single maternal nicotine dose administered on gestational day 20, but had a smaller effect on nicotine distribution to brain after continuous nicotine infusion (Keyler et al 2003 & 2005). The nicotine-specific antibody concentration in foetal serum was 10% (foetal brain 1%) of that in maternal serum. Overall these data support the idea that vaccination against nicotine reduces distribution of nicotine to the brain by sequestering it in the serum.

Due to the mislabelling of conjugates (see Chapter 2) antibody titres in the rats immunised against cotinine were relatively low (~1:3,000 for the repeated nicotine injections, and ~1:800 in the chronic administration study; see Figure 2.10 in Chapter 2), compared to those achieved in the studies of anti-nicotine vaccines ($\geq 1:10,000$). This could explain why there wasn't a greater effect of antibodies on cotinine levels, especially in the brain. Based on the findings of

the anti-nicotine vaccine studies, a significant decrease in cotinine concentration should have been observed in the brains of animals immunised against cotinine, rather than just a “lack of increase”, as anti-cotinine antibodies should sequester cotinine in the blood and prevent it from entering the brain. The elevation in brain cotinine levels observed in our first experiment (see Figure 3.1) could have been due to contamination of the brain samples with blood during dissection procedures. In order to address this issue, rats were perfused with PBS after the chronic infusion study in an attempt to remove as much blood as possible from the brain and surrounding tissues. Although brain cotinine levels were again slightly higher in vaccinated rats in this study, the difference was no longer significant. Some contamination with minor traces of blood may have remained even after perfusion, which could account for some of the cotinine measured in these brain samples. Serum cotinine levels were found to be very high in the second study, so even a small amount of blood could have had an impact on brain cotinine levels measured.

3.3 Radioligand binding

3.3.1 Introduction

Nicotine addiction is characterised by the repeated administration of nicotine through cigarettes or other tobacco products, resulting in sustained blood nicotine levels. The effects of chronic exposure to nicotine and other nicotinic agonists on nAChR levels have been studied both in vitro and in vivo using a variety of radioligands.

High-affinity binding of [³H]nicotine and [³H]cytisine has been correlated with nAChRs comprised of $\alpha 4$ and $\beta 2$ subunits (Whiting et al 1991, Flores et al 1992, Zoli et al 1998), a subtype that appears to be particularly easily upregulated by nicotine, possibly due to its high affinity compared to other receptor subtypes (Perry et al 1999, Nguyen et al 2003). [³H]epibatidine on the other hand also labels a number of other subtypes in addition to $\alpha 4\beta 2$ nAChRs, including $\alpha 3\beta 2^*$ and $\beta 4^*$ receptors (Houghtling et al 1995, Ridley et al 2001, Nguyen et al 2003, Xiao & Kellar 2004, Marks et al 2006). Binding of [¹²⁵I] α Bgt and [³H]methyllaconitine ([³H]MLA) correlates with the $\alpha 7$ subunit, however upregulation of this subtype is less robust than that observed for [³H]nicotine

binding sites and requires higher nicotine concentrations that may not be relevant to human smokers (Wonnacott 1990, Pauly et al 1991, Barrantes et al 1995, Peng et al 1997, Orr-Urtreger et al 1997, Davies et al 1999, Whiteaker 1999).

Several groups have demonstrated that in cells stably expressing defined heterologous nAChR subtypes, incubation with nicotine for several hours to several days can increase the density of nAChR binding sites corresponding to the $\alpha 4\beta 2$, $\alpha 3\beta 2$, and $\alpha 3\beta 4$ subtypes, as well as several others (Peng et al 1994 & 1997, Bencherif et al 1995, Gopalakrishnan et al 1996 & 1997, Wang et al 1998, Whiteaker et al 1998, Meyer et al 2001). For example, comparing 6 different nAChR subunit combinations, Xiao and Kellar observed that culturing HEK 293 cells in the presence of nicotine (0.2 - 100 μ M) for 5 days resulted in a concentration-dependent increase in [3 H]epibatidine binding in membrane preparations from all transfected cell lines (Xiao & Kellar 2004). However, the extent of the increase varied markedly depending on the subtype of rat nAChR expressed, with the largest increases seen in subtypes containing the $\beta 2$ subunit ($\alpha 3\beta 2 > \alpha 2\beta 2 > \alpha 4\beta 2$) rather than the $\beta 4$ subunit.

In cell lines, upregulation of surface nAChRs by nicotine was shown to include both $\alpha 3^*$ (Meyer et al 2001, Ridley et al 2001) and $\alpha 6^*$, as well as $\alpha 4^*$ (Nelson et al 2003) and $\alpha 7$ receptors (Quik et al 1996).

Chronic administration of nicotine (by intravenous or subcutaneous infusion, administration in drinking water or through repeated injections) elicits a dose-dependent increase in the density of [3 H]nicotine and [125 I] α Bgt binding sites in the brains of rodents (Wonnacott 1990, Flores et al 1992, Rowell & Li 1997).

This upregulation is reversible, and after discontinuation of nicotine treatment, [3 H]nicotine binding levels return to control values within 7-10 days in mice (Marks et al 1985) and 15-20 days in rats (Collins et al 1990), whereas [125 I] α Bgt binding returns to control levels within 2-4 days in both rat and mouse (Miner & Collins 1989, Collins et al 1990). In contrast, α -conotoxin MII-sensitive $\alpha 6^*$ nAChR subtypes appear to decrease in mouse striatum (Lai et al 2005) but increase in rat brain (Parker et al 2004) as a consequence of nicotine administration.

Both in vivo and in vitro the upregulation of binding sites by nicotine reflects an increase in total receptor number (B_{\max}) rather than a change in affinity (K_D) (Wonnacott 1990, Gopalakrishnan et al 1997) and does not appear to involve an increase in nAChR subunit mRNA (Marks et al 1992, Peng et al 1994, Bencherif et al 1995), indicating that upregulation occurs through a post-transcriptional mechanism.

Humans self-administer nicotine in the form of cigarettes and other tobacco products. Significant increases in [^3H]nicotine, [^3H]cytisine, and [^3H]epibatidine binding are seen in human post mortem brain tissue (cortex, cerebellum, hippocampus & thalamus) of smokers, compared to non-smokers (Benwell et al 1988, Breese et al 1997, Perry et al 1999). In white blood cells from smokers, [^3H]epibatidine binding is also significantly increased compared to non-smoking controls, but there is no difference in [^{125}I] α Bgt binding (Cormier et al 2004). As in rodents, the upregulation of nAChRs by nicotine appears to be due to an increase in total receptor number, rather than receptor affinity, and is dose-dependent. Receptor levels in the brains of ex-smokers who quit at least 2 months before death are comparable to those found in non-smoking subjects, indicating that smoking-induced changes in nAChR numbers are reversible after cessation of nicotine intake (Breese et al 1997).

In order to determine whether immunisation against cotinine has any effect on nicotine-induced receptor upregulation, we performed radioligand binding assays with [^3H]epibatidine and [^{125}I] α -Bungarotoxin on brain homogenates from rats after various nicotine administration schedules.

3.3.2 Methods

3.3.2.1 Membrane preparation

Rats were culled by cervical dislocation or transcardiac perfusion (see section 3.2.2.2.2). Brains were quickly removed, and hemispheres frozen in liquid nitrogen and stored at -20°C until use.

Brain membranes were prepared as previously described (Davies et al 1999, Sharples et al 2000). Hemispheres (minus cerebellum) were homogenised in ice-cold 0.32 M sucrose buffer (containing 1 mM EDTA, 0.01% NaN_3 (pH 7.4, 10% w/v) 0.1 mM PMSF; pH 7.4). The homogenate was centrifuged ($15,000 \times$

g) for 30 min at 4°C to give a membrane pellet (Beckman Avanti J-25 centrifuge with JA25-50 rotor). The pellet was resuspended in 50 mM phosphate buffer (40 mM K₂HPO₄, 10 mM KH₂PO₄, 1 mM EDTA, 0.01% w/v NaN₃, 0.1 mM PMSF; pH 7.4), and again centrifuged as above. The wash step was repeated, and the final pellet was resuspended in ice-cold 50 mM phosphate buffer (2.5 ml/g tissue). Aliquots were then frozen until use.

Protein concentration was estimated using a colorimetric protein dye reagent (Bradford 1976).

3.3.2.2 [³H]epibatidine binding

[³H]epibatidine binding was carried out as previously described (Sharples et al. 2000). Membrane preparations (final concentration 1 mg/ml) were added to assay buffer (118 mM NaCl, 4.8 mM KCl, 2.5 mM CaCl₂·2H₂O, 2 mM MgSO₄, 20 mM HEPES, 20 mM Tris, 0.1 mM PMSF, 0.01% w/v NaN₃; pH 7.4), containing [³H]epibatidine (54 Ci/mmol; Amersham Biosciences, Buckinghamshire, UK; final concentration 1 nM) to give a total volume of 1 ml. Non-specific binding was determined in the presence of 1 mM nicotine. Samples were incubated at room temperature for at least 1 h, before being chilled to 4°C for at least 30 min. Then samples were filtered at 4°C through a double thickness of Gelman GFA filters (pre-soaked overnight at 4°C in 0.3% polyethylene imine (PEI)), using a Brandel cell harvester. Filters were washed 3 times with ice cold PBS (150 mM NaCl, 8 mM K₂HPO₄, 10 mM KH₂PO₄, 0.01% w/v NaN₃; pH 7.4). Filter discs were then counted for radioactivity using a Packard 1600 Tricarb scintillation counter (efficiency ~ 45%) and liquid scintillation cocktail (MicroscintTM 40, Packard Bioscience Company, Groningen, Netherlands).

3.3.2.3 [¹²⁵I]αBungarotoxin binding

[¹²⁵I]-α-bungarotoxin ([¹²⁵I]αBgt) binding was carried out according to Whiteaker et al 2000 with some modifications. Membranes (final concentration 0.5 mg/ml) and [¹²⁵I]αBgt (Amersham Biosciences, Buckinghamshire, UK; final concentration 10 nM) were diluted in 50 mM phosphate buffer (see section 3.3.2.1) supplemented with 0.1% BSA (final volume 100 µl). Non-specific binding was determined in the presence of 1 mM nicotine. Samples were

incubated for ~3 h at 37°C. Then 1 ml of buffer was added to each tube, and samples were incubated for a further hour at 37°C. Samples were cooled to 4°C for at least 30 min before filtration through two layers of Gelman GFA filters (pre-soaked overnight at 4°C in 0.3% PEI and 4% milk powder, respectively), using a Brandel cell harvester. Filters were then washed and counted as described for [³H]epibatidine binding (see section 3.3.2.2). Counting efficiency for this assay was ~60%.

3.3.2.4 Data Analysis

Data shown are means ± S.E.M for each group. Initially, a 2-way ANOVA (treatment x vaccination) was used to examine the data. Where significant differences were found, unpaired t-tests were performed to follow up.

3.3.3 Results

3.3.3.1 Sub-chronic intermittent nicotine administration (4 days)

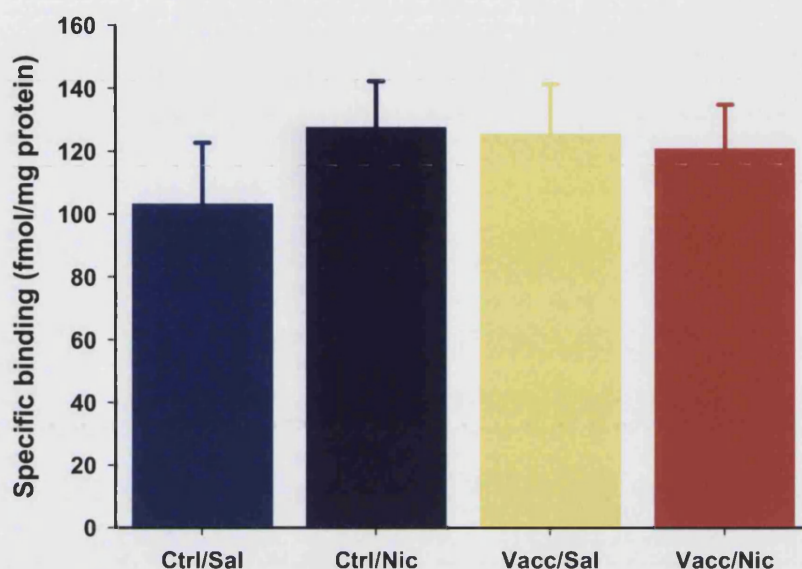
As part of the locomotor experiments (see section 4.2.2.4), rats had been given daily injections of nicotine (0.5 mg/kg) or saline for 4 days. On day 5 all rats received a saline injection. After completing the last locomotor session, they were sacrificed and the brains harvested for radioligand binding. As a result, binding was effectively measured after one day of nicotine withdrawal.

[³H]epibatidine binding:

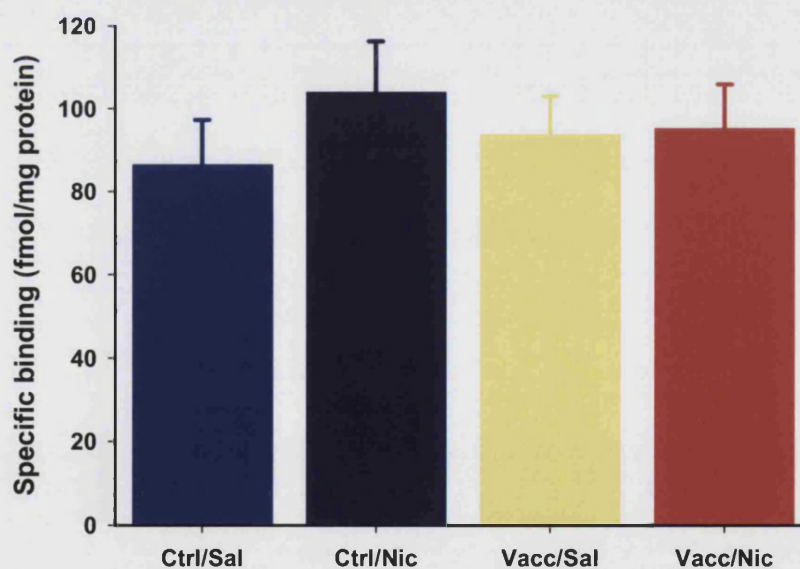
Non-specific binding accounted for ~17% of total [³H]epibatidine binding. For all four groups specific binding averaged around 100 – 125 fmol/mg protein (see Figure 3.3 A). There was no evidence of upregulation of nicotinic binding sites after nicotine administration, although a small trend in this direction was observed in the groups immunised with TT-Cysteine (control vaccine). There were no significant differences in [³H]epibatidine binding levels between any of the treatment groups.

Figure 3.3: Effects of vaccination on [³H]epibatidine and [¹²⁵I]- α -bungarotoxin binding after 4-day nicotine administration

A



B



Rats were given a daily injection of nicotine (0.5 mg/kg) or saline for 4 days as part of locomotor experiments (see section 4.2.2.4). On day 5 all rats received saline; they were culled and brain membranes prepared.

Brain homogenates were incubated with 1 nM [³H]epibatidine (A; see section 3.3.2.2) or 10 nM [¹²⁵I]- α -Bungarotoxin (B) (see section 3.3.2.3) and specific binding determined (expressed as fmol/mg protein).

Data shown are means \pm S.E.M ($n = 12$).

[¹²⁵I]αBgt binding:

Non-specific binding accounted for ~63% of total [¹²⁵I]αBgt binding. For all four groups specific binding was approximately 85 – 105 fmol/mg protein (see Figure 3.3 B). There was no sign of nicotinic binding site upregulation in any of the groups, and no significant differences were observed between any of the groups.

There are two possible explanations for the lack of upregulation of nicotinic binding sites: either the nicotine treatment schedule was not sufficient to cause upregulation of nAChRs, or any upregulation that had occurred could no longer be detected due to reversal of the effect during the one day of nicotine withdrawal. We therefore decided to look at [³H]epibatidine binding levels after a longer period of daily nicotine administration.

3.3.3.2 Chronic intermittent nicotine administration (15 days)

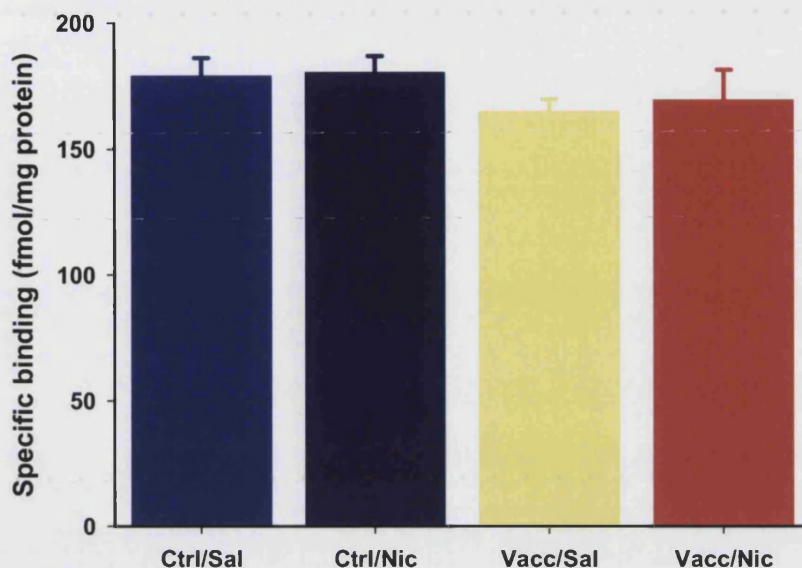
As part of the locomotor experiments in Trial 3 (see section 4.2.2.5), rats were injected daily with either nicotine (0.5 mg/kg) or saline for 15 consecutive days and were culled 5 min after receiving their final dose.

Non-specific binding accounted for ~18.5% of total [³H]epibatidine binding. For all four groups specific binding averaged around 170 – 180 fmol/mg protein (see Figure 3.4).

Despite the longer exposure to nicotine compared to Trial 2, no significant nAChR upregulation was observed in the nicotine-treated groups, and there were no significant differences in [³H]epibatidine binding between any of the treatment groups (see Figure 3.4).

As daily nicotine injections did not appear to be sufficient to cause nAChR upregulation, we decided to examine [³H]epibatidine binding after chronic nicotine infusion.

Figure 3.4: Effects of vaccination on [³H]epibatidine binding after 15-day nicotine treatment



Rats were given daily nicotine (0.5 mg/kg) or saline injections as part of locomotor experiments for 15 days. Animals were culled 5 min after their last injection.

Brain homogenates were incubated with 1 nM [³H]epibatidine (see section 3.3.2.2) and specific binding determined (expressed as fmol/mg protein).

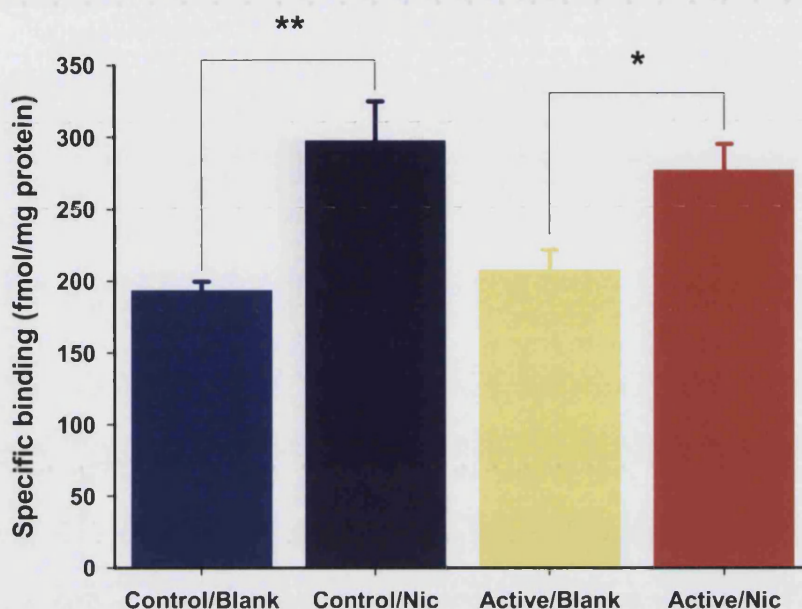
Data shown are means \pm S.E.M (n = 6).

3.3.3.3 Chronic nicotine infusion (7-day OMP)

In Trial 5, rats received a chronic infusion of nicotine (3.16 mg/kg/day) for 7 days via subcutaneous osmotic minipumps as part of the mecamylamine-precipitated nicotine withdrawal experiments (see section 4.5.2.3). Control rats were implanted with a "blank". Having completed the behavioural experiments, rats were perfused transcardially with PBS (see section 3.2.2.2.2) to enable the determination of brain nicotine and cotinine levels (see section 3.2) without blood contamination.

Non-specific binding accounted for ~33% of total [³H]epibatidine binding. For both saline-treated groups (control and active vaccine) specific binding averaged around 200 fmol/mg protein (see Figure 3.5), while in the two nicotine-treated groups specific [³H]epibatidine binding levels were approximately 260 – 300 fmol/mg protein.

Figure 3.5: Effects of vaccination on [³H]epibatidine binding after 7-day chronic nicotine infusion



Rats received a chronic infusion of nicotine (3.16 mg/kg/day) for 7 days via osmotic minipumps as part of the mecamlamine-precipitated nicotine withdrawal experiments (see section 4.5.2.3). Control rats were implanted with a "blank". After the behavioural experiments, rats were perfused transcardially with PBS (see section 3.2.2.2.2), and brain homogenates prepared.

Brain homogenates were incubated with 1 nM [³H]epibatidine (see section 3.3.2.2) and specific binding determined (expressed as fmol/mg protein).

Data shown are means \pm S.E.M ($n = 6-8$). * $p < 0.05$, ** $p < 0.01$

A 2-way ANOVA revealed a significant effect of nicotine treatment ($p < 0.0001$), but no vaccine effect ($p = 0.7378$). Unpaired t-tests revealed significant increases in [³H]epibatidine binding after nicotine treatment: specific binding was increased by 54% for the group immunised with TT-Cysteine (control vaccine; $p = 0.0011$) and by 31% for the active vaccine group (TT-CotSH; $p = 0.0203$), compared to their respective controls (see Figure 3.5). The differences between nicotine-treated control animals and those immunised against cotinine, however, were not significant.

3.3.4 Discussion

Binding methods:

Radioligand binding on whole brain homogenates is a relatively quick and simple method used to examine the effect of nicotine administration on nicotinic binding sites. It provides an overall measure of the mean change in binding sites across the entire brain; however, it is not possible to determine whether such changes are uniform throughout the tissue or only restricted to certain brain structures. In order to build up a more detailed picture it is possible to perform binding studies on individual brain structures, although the small quantity of tissue obtained from an individual animal can be a limiting factor with this approach.

Autoradiography on brain sections is an alternative option, which can be used to define brain regions enriched (or depleted) in particular binding sites. This technique enables the systematic scanning of the entire brain for changes in binding sites and provides much greater resolution than radioligand binding on whole brain homogenates; however, it is also much more complex and time-consuming to perform, and even with this technique it not possible to determine cellular or subcellular localisation of binding sites.

Two main classes of nAChR have been identified in mammalian brain using receptor binding methods: one is selectively labelled by [¹²⁵I]αBungarotoxin (see Figure 3.6) and correlates with the α7 homomeric subtype (Orr-Urtreger et al 1997); the other has higher affinity for acetylcholine, nicotine, and cytisine (Nguyen et al 2003). There are several different subtypes of these high-affinity nAChRs: the major form is thought to be α4β2 (Flores et al 1992), of which multiple forms may exist based on variable stoichiometry or the inclusion of additional subunits such as α5. A second form closely resembles ganglionic nicotinic receptors, believed to be α3β4*. This subtype may also exist in several variations, with additional subunits or variable stoichiometry. A third subtype of high-affinity nAChRs consists of a heterogeneous mix of subunits: α6 (and/or α3) paired with β2 subunits, with the possible addition of β3 or α4 subunits in some cases (Le Novère et al 1999, Lena et al 1999, Quik et al 2000, Klink et al 2001, Azam et al 2002, Champtiaux et al 2002, Whiteaker et al 2002, Marubio et al 2003).

Figure 3.6: Binding affinities of nicotinic radioligands

| Radioligand | K _d (nM) | | | |
|------------------------------|---------------------|----------|-----------|------------|
| | α4β2* | α7 | α3β4 | α3β2* |
| [³ H]nicotine | 0.89-9 | - | - | - |
| [³ H]cytisine | 0.15-0.96 | - | - | - |
| [³ H]epibatidine | 0.011-0.086 | - | 0.306-4.9 | 0.013-0.14 |
| [¹²⁵ I]αBgt | - | 0.4-1.66 | - | - |

Binding affinities derived from saturation binding experiments on brain membranes / primary cultured neurones (α4β2 & α7) or heterologously expressed nAChRs.

(adapted from Sharples & Wonnacott 2001)

The alkaloid (±)-epibatidine (from the skin of the Ecuadorian frog *Epipedobates tricolor*) is a very high-affinity agonist at most nAChRs (see Figure 3.6; Parker et al 1998, Xiao et al 1998, Zoli et al 2002, Champtiaux et al 2003). After initially measuring both [³H]epibatidine and [¹²⁵I]αBgt binding, we focussed on [³H]epibatidine binding as an indicator of nAChR upregulation, as epibatidine-binding nAChR subtypes are generally more readily upregulated than the α7 receptor subtype. We used whole brain homogenates for the radioligand binding experiments, since epibatidine labels a number of nAChR subtypes, and any upregulation occurring was therefore likely to be widespread and readily detectable even in whole brain homogenates.

Upregulation after repeated daily injections of nicotine:

Chronic nicotine administration, by means of repeated injections or continuous infusion (i.v. or s.c.), has been shown to cause upregulation of nAChR (Wonnacott 1990). This increase in nAChRs by long-term nicotine administration in the rat may strongly depend on the dosage and mode of delivery of nicotine, as well as on the rat strain used (Parker et al 2004). We did not observe any upregulation in [³H]epibatidine or [¹²⁵I]Bgt binding sites after intermittent nicotine treatment by daily injection (0.5 mg/kg) for 4 days (see Figure 3.3 A & B). Ksir et al showed increases of 18 - 26% in [³H]acetylcholine binding levels in the cerebral cortex of rats injected subcutaneously with nicotine doses of 0.1 - 0.4 mg/kg/day (Ksir et al 1985). Several other groups

have also shown that nAChRs are upregulated following repeated nicotine administration, however, these studies have utilised twice-daily (or more frequent) injections or a continuous infusion of nicotine (Wonnacott 1990). It seems likely that with our once-daily injection regime the plasma nicotine levels achieved were too low for most of the day to result in a measurable upregulation of receptors.

Examining a range of doses (0.6 - 4.8 mg/kg/day), Rowell & Li observed that with a twice-daily injection schedule, 2.4 or 4.8 mg/kg/day (total) for 10 days produced significant upregulation of [^3H]cytisine binding sites in all brain areas examined (Rowell & Li 1997). Our dose of 0.5 mg/kg is much lower than this, and although we administered nicotine for 15 days (instead of 10) in our second experiment (see section 3.3.3.2), we did not observe any upregulation of [^3H]epibatidine binding sites (see Figure 3.4). Jacobs et al found only a relatively small increase in binding by 15-25% in all brain areas examined, following administration of 2 mg/kg nicotine tartrate twice daily for 10 days (Jacobs et al 2002). It is therefore possible that the dose used in our first two studies was just not large enough to produce a substantial increase in binding sites.

Others have shown nicotine-induced nAChR upregulation using somewhat higher doses: twice daily injections of 1 mg/kg nicotine produced significant upregulation at 5 days, which increased further by 10 and 21 days (Schwartz & Kellar 1985). No change in binding was observed after just one day of nicotine administration. In rats treated with nicotine for 10 days, upregulation was significantly reduced, though still present, after 7 days of nicotine withdrawal.

Upregulation following chronic nicotine administration:

Mugnaini et al have shown that chronic infusion of rats with 3 mg/kg/day of nicotine for 14 days resulted in higher levels of [^3H]nicotine binding in 11 out of 15 brain regions analysed, but that [^3H]MLA binding was increased in only 4 regions (Mugnaini et al 2002). Using autoradiography, Nguyen et al observed a significant upregulation of $\alpha 4\beta 2$ -like binding in rat brain after chronic nicotine infusion for 14 days (6 mg/kg/day, s.c.), with relatively little effect on $\alpha 3/\alpha 6\beta 2$ -like and $\alpha 3\beta 4$ -like binding (Nguyen et al 2003). This upregulation also showed considerable regional variation across the 33 brain regions studied. These

doses are somewhat higher than those used in our chronic infusion protocol; however Rowell & Li observed that chronic subcutaneous infusion of 2.4 or 4.8 mg/kg/day of nicotine for 10 days resulted in a significant increase in [³H]cytisine binding sites in all 3 brain areas studied, whereas at their 1.2 mg/kg/day dose, upregulation was only seen in the cortex. The doses used in this study were more comparable to ours, and the upregulation observed is largely in agreement with the increase in binding we observed at 3.16 mg/kg/day for 7 days (see Figure 3.5).

Role of nAChR upregulation in nicotine dependence:

After an initial peak in nicotine levels following a subcutaneous injection, blood nicotine concentrations decline rapidly due to the short half-life of nicotine (Rowell & Li 1997), and the same is also true for a human smoking a cigarette (see Chapter 1). Initially, nicotine entering the blood stream activates nAChRs, but with prolonged exposure the receptors desensitise (see section 1.3.2), decreasing the impact of subsequent cigarettes smoked. Although it is not clear exactly how nAChR desensitisation is linked to receptor upregulation, it has been hypothesised that nAChRs are turned over in the cell membrane more slowly when they are in a desensitised conformation, leading to an overall increase in the number of receptors in the cell membrane (Dani & De Biasi 2001). When nicotine is removed from the brain, for example by overnight abstinence, the receptors recover from desensitisation, resulting in an excess of excitable receptors available for stimulation. This hyperexcitability is thought to contribute to the nicotine withdrawal symptoms experienced by smokers attempting to quit. After a night of abstinence, nicotine from the first cigarette is able to activate the re-sensitised receptors, stimulating dopamine release in the reward circuitry (see Chapter 1), which provides further reinforcement. By continued smoking, more and more of the excess nAChRs are gradually desensitised towards a more "normal" level of active receptors (Dani & De Biasi 2001). This could explain why smokers often consider the first cigarette of the day the most pleasurable (Russell 1989).

Effect of vaccination against cotinine:

Cotinine is thought to antagonise the actions of nicotine through its weak agonist activity at nAChRs. Vaccination against cotinine should remove this antagonism by raising antibodies that bind cotinine and prevent it from entering the brain. This should lead to a potentiation of nAChR upregulation in nicotine-treated animals, resulting in upregulation at relatively low nicotine doses, which do not usually produce upregulation, or a general increase in levels of upregulation observed in vaccinated rats treated with nicotine, compared to the control vaccinated nicotine-exposed group. However, we did not observe any effect of vaccination on the upregulation of [³H]epibatidine binding sites after chronic nicotine exposure (see Figure 3.5), the only treatment schedule that produced upregulation in the nicotine-treated groups. It is possible that higher anti-cotinine antibody titres than those achieved in our studies (see Figure 2.10) are required to produce a measurable effect on nAChR upregulation.

3.4 Chapter conclusions

Having established that immunisation with TT-CotSH resulted in the production of cotinine-specific antibodies, we went on to look at whether these anti-cotinine antibodies could impact on the distribution of cotinine in rats.

Nicotine and cotinine levels in both brain and blood were dependent on the dose of nicotine administered. Anti-cotinine vaccination had no impact on nicotine concentrations measured in plasma or brain, providing further evidence for the specificity of the antibodies raised in response to vaccination. We were able to demonstrate a significant increase in plasma cotinine levels in animals immunised against cotinine, compared to controls, after both intermittent and continuous nicotine administration. This supports the hypothesis that anti-cotinine antibodies retain cotinine in the bloodstream and prevent it from crossing the blood-brain-barrier. Although we also observed an increase in the level of brain cotinine in the vaccinated group in our first study, this was most likely due to contamination of the brain samples with blood during dissection. By perfusing rats prior to dissection for the second study, the likelihood of

contamination was reduced and the brain cotinine levels of vaccinated and control groups were no longer significantly different.

Since prolonged administration of nicotine has been shown to result in upregulation of nAChRs, we also examined whether removing the antagonism by cotinine through immunisation would have any effect on receptor upregulation, as measured by radioligand binding.

We did not observe any significant increase in [^3H]epibatidine or [^{125}I] αBgt binding levels as a result of intermittent administration of nicotine for 4 or 15 days, possibly due to the low dose of nicotine used compared to some other studies. However, chronic infusion of nicotine (3.16 mg/kg/day) for 7 days did lead to a significant upregulation of nicotinic receptors. Immunisation against cotinine should have enhanced the level of nAChR upregulation as a consequence of removing cotinine-mediated antagonism of nicotine's actions; however no vaccine effects were observed. This may have been due to the low anti-cotinine antibody titres achieved in Trial 5 as a result of an error in the labelling of conjugate vials (see Chapter 2).

Upregulation of nAChRs has been shown to occur in human smokers (Benwell et al 1988, Breese et al 1997, Perry et al 1999) and appears to be dose-dependent, as there is a positive correlation between the level of upregulation and the number of packs of cigarettes smoked per day. This indicates that the increase in nicotinic binding sites is related to the blood and brain concentrations of nicotine achieved during smoking.

It has been hypothesised that having an increased number of nAChRs may lead to heightened or abnormal potentiation of ordinary synaptic activity during times of abstinence, contributing to the agitation and discomfort of withdrawal symptoms, which often cause abstinent smokers to relapse. NRT aims to provide a low level of nicotine to help alleviate such withdrawal symptoms, so reducing the antagonism by cotinine through cotinine-specific antibodies (as a result of vaccination) should make NRT more effective.

In summary we have shown that, as a consequence of anti-cotinine vaccination, cotinine is sequestered in the blood and prevented from entering the brain by cotinine-specific antibodies, without affecting the distribution of nicotine. The removal of cotinine-mediated antagonism in this manner should enhance the effects of nicotine, here measured in terms of upregulation of nAChR after prolonged nicotine administration. Whilst we were able to demonstrate upregulation of nicotinic receptors after chronic nicotine treatment, the low antibody titres achieved in this trial prevented us from ruling out any vaccine-related effects on binding levels.

Chapter 4

**Effects of vaccination
on behavioural
measures of nicotine
dependence**

Chapter 4

Effects of vaccination on behavioural measures of nicotine dependence

4.1 Chapter Introduction

Addiction is a complex behavioural phenomenon with causes and effects ranging from molecular mechanisms to social interactions. Over time, the initial molecular interactions, which alter the activity and metabolism of neurons sensitive to the drug, lead to changes in the properties of individual neurons and circuits, resulting in the expression of complex behaviours such as dependence, tolerance, sensitisation, and craving (Mansvelder & McGehee 2002, Laviolette & van der Kooy 2004).

As discussed in Chapter 3, physiologically relevant nicotine concentrations have been shown to upregulate $\alpha 4\beta 2^*$ receptors (Schwartz & Kellar 1985, Flores et al 1992, Buisson & Bertrand 2001). In correlation with this upregulation of nicotinic binding sites following nicotine pre-exposure, several laboratories have also reported increases in various functional and behavioural responses (Ksir et al 1987, Clarke et al 1988, Rowell & Wonnacott 1990, Yu & Wecker 1994, Buisson & Bertrand 2001). For example, pre-exposure to nicotine appears to sensitise rats to its locomotor and self-administration effects (Clarke & Kumar 1983, Walter & Kuschinsky 1989, Shoaib et al 1997), and upregulation of nicotinic binding sites has been reported after nicotine self-administration (Donny et al 2000).

Reinforcing and subjective stimulus effects of nicotine

Nicotine is known to elicit rewarding effects (positive reinforcement) in humans and other species, and stimulus properties of nicotine are thought to control nicotine-taking behaviour in both humans and laboratory animals (Stolerman & Shoaib 1991, Rose & Corrigall 1997). The reinforcing stimulus effects of nicotine can be assessed using the intravenous self-administration technique (see section 4.3). This is considered to be the animal model of nicotine-taking behaviour that comes closest to the situation of the human smoker, because of

the pattern of nicotine administration (frequent small doses) and the fact that nicotine intake is "voluntary" and based on the positive reinforcing effects of the drug. The technique is therefore frequently used for studying tobacco dependence and evaluating potential anti-smoking medications (Rose & Corrigall 1997).

The ability to perceive and identify the subjective effects of a drug is considered important in addiction, as it encourages the development of drug-seeking behaviour and directs it towards one substance rather than another. It is widely believed that humans abuse psychoactive drugs in order to obtain their characteristic subjective effects (Stolerman & Shoaib 1991).

Like many addictive drugs, nicotine also has potent, aversive, unpleasant effects, with many smokers experiencing noxious effects such as nausea, coughing and dizziness on their initial exposure to tobacco. However, with repeated exposure to the drug, tolerance to these aversive effects develops (Laviolette & van der Kooy 2004). Aversive stimulus effects of nicotine are often examined using conditioned taste aversion procedures (see section 4.4), where rats learn to avoid a flavoured solution when its consumption is previously paired with administration of nicotine (Kumar et al 1983, Iwamoto & Williamson 1984). Aversive effects have been implicated in the regulation of nicotine intake, and may play a role in setting an upper limit to the amount consumed (Shoaib et al 2003).

Behavioural sensitisation to the effects of nicotine

Neurochemical aspects: Nicotine has the ability to enhance basal neuronal firing and/or basal transmitter release in the mesolimbic dopamine pathway, as do other drugs of abuse (Andreoli et al 2003). Adaptations occurring in brain reward circuits as a result of excessive stimulation are thought to contribute to the development of the compulsive drug use, which characterises addiction. Upregulation of nAChRs and sensitisation of dopamine release in the striatum are two examples of adaptations occurring in the brain as a consequence of prolonged exposure to nicotine, which are discussed in more detail in Chapters 2 & 3.

Environmental context: Environmental or contextual stimuli can become associated with the effects of nicotine if exposure to the drug is repeatedly paired with such “conditioned stimuli” (Bevins & Palmatier 2003). Over time these conditioned stimuli can come to control responses related to drug seeking, withdrawal, and dependence.

In animals pre-treated with nicotine, sensitisation to the effects of a subsequent nicotine challenge on locomotor activity (see section 4.2) and nucleus accumbens dopamine release has been observed. However, this sensitisation appears to be more pronounced in animals that were repeatedly administered the drug in the testing environment during nicotine pre-treatment, suggestive of an emerging association between the conditioned stimulus (context) and drug effects (Walter & Kuschinsky 1989, Reid et al 1996, Bevins et al 2001). Stimuli associated with nicotine self-administration have also been shown to reinstate nicotine seeking in rats (Liu et al 2006).

Such associative learning is thought to resemble the strong associations smokers develop between environmental cues associated with the act of smoking and the reinforcing effects of nicotine (Stolerman & Shoaib 1991, Geier et al 2000, Laviolette & van der Kooy 2004). Such smoking-related environmental stimuli, including the sight, smell and taste of cigarettes, contexts in which smoking occurred, or behaviours that frequently accompanied smoking (handling a cigarette, for example), have been implicated as an important factor in triggering relapse in abstinent tobacco smokers (Balfour et al 2000, Caggiula et al 2001, Liu et al 2006).

Effects of nicotine abstinence/withdrawal

Positive reinforcement plays a major role in the acquisition and routine maintenance of nicotine self-administration, both in smokers and in animal models. During nicotine withdrawal, for example during a quit attempt in smokers, a nicotine abstinence syndrome develops, with typical symptoms including irritability, anxiety, depression, difficulty concentrating, and craving for tobacco (Malin 2001). An equivalent nicotine abstinence syndrome has also been studied in rodents rendered nicotine-dependent by means of continuous nicotine infusion; abstinence is induced by termination of the infusion (spontaneous abstinence) or through the administration of a nicotinic

antagonist, such as mecamylamine (mecamylamine-precipitated withdrawal, see section 4.5). The resulting symptoms of nicotine withdrawal can be alleviated by administration of nicotine. This mirrors the human situation, where smoking serves as a "negative reinforcer" by providing relief from the aversive state, which explains why the desire to alleviate the discomforts of tobacco withdrawal is the most commonly cited reason for relapse during a smoking quit attempt (Malin 2001).

Nicotine replacement therapy (NRT) reduces the severity of withdrawal symptoms and cravings by providing a continuous low-dose infusion of nicotine. It can approximately double the success rate of a quit attempt, and is the most commonly used medication for smoking cessation (Schneider et al 2001, Hughes 2003, Foulds et al 2004). However, even with NRT, the majority of quit attempts are still unsuccessful. The anti-cotinine vaccine studied in this thesis is aimed at making NRT more effective by removing or reducing any cotinine-mediated antagonism of nicotine's actions.

In this chapter we studied the effects of anti-cotinine vaccination on a wide variety of behavioural effects of nicotine. In an attempt to cover as many aspects of nicotine addiction and smoking cessation as possible, we utilised animal models of drug-taking, drug-seeking, and drug-withdrawal behaviour, as well as examining locomotor depressant/stimulant properties of nicotine, its reinforcing and subjective stimulus effects, and the role of environmental stimuli on nicotine-induced behavioural measures.

4.2 Effects of anti-cotinine vaccination on nicotine-induced & conditioned locomotor activity

4.2.1 Introduction

Effects of acute nicotine on locomotor activity

In experimentally naive rats, systemic nicotine administration produces a decrease followed by an increase in locomotor activity in a dose-dependent manner (Clarke & Kumar 1983, Ksir et al 1987, Walter & Kuschinsky 1989, Bevins et al 2001, Bevins & Palmatier 2003). Both the initial reduction and the increase in locomotor activity are blocked by administration of the centrally

active, non-selective nicotinic antagonist mecamylamine in a dose-related manner (Clarke & Kumar 1983, Walter & Kuschinsky 1989).

Dopaminergic reward pathways (see Chapter 1) appear to play a major role in the effects of nicotine on locomotor activity: administration of nicotine directly into the VTA has been shown to enhance locomotor activity in rats, as well as increasing accumbal dopamine release, whereas injection directly into the NAcc also increased accumbal dopamine output, but had no effect on locomotion (Ferrari et al 2001). Further evidence for the involvement of dopaminergic pathways in nicotine-induced locomotor activity comes from lesion studies, which have found that lesions of mesolimbic DA neurons attenuate the locomotor stimulant properties of nicotine (Benwell & Balfour 1992, Louis & Clarke 1998).

Effects of chronic nicotine on locomotor activity

Chronic nicotine treatment produces tolerance to the initial locomotor depressant effects and sensitisation to the subsequent locomotor stimulatory effects of nicotine in rats (Clarke & Kumar 1983, Ksir et al 1987, Clarke et al 1988, Walter & Kuschinsky 1989, Vezina et al 1992, Shoaib & Stolerman 1992, Whiteaker et al 1995, Reid et al 1996, Bevins et al 2001), with tolerance to the depressant effects persisting for at least 3 weeks (Clarke & Kumar 1983). Sensitisation to the locomotor stimulant effects of nicotine can be observed after as few as 5 days of nicotine pre-treatment (Ksir et al 1985 & 1987, Reid et al 1996), and is dose-dependent. The locomotor response to a 0.2 mg/kg challenge dose of nicotine did not differ from saline controls in rats pre-treated with 0.1 mg/kg/day nicotine for 5 days, but groups pre-treated with higher doses showed increased stimulation of locomotor activity in response to the same test dose. Pre-treatment with 0.2 mg/kg for the same time period shifted the bell-shaped dose-response curve to a nicotine challenge (0.1 – 1.6 mg/kg) upwards, so that greater stimulation was observed at each test dose after nicotine exposure, compared to baseline activity in response to the test dose measured prior to the 5 day pre-treatment (Ksir et al 1987). The enhanced locomotor activity resulting from 5 days of nicotine administration (0.2 mg/kg) was even observed after 7 days of withdrawal, but had returned to saline levels after 21 days (Ksir et al 1985). Sensitisation to the locomotor stimulant effect of an acute

nicotine challenge (0.2 mg/kg) was also seen after subcutaneous infusion of nicotine (1.5 mg/kg/day) by osmotic minipump for up to 14 days (Fung & Lau 1988).

Effects of conditioning on nicotine-induced locomotor activity

This sensitisation to a nicotine challenge observed in nicotine-treated rats in terms of both locomotor activity and nucleus accumbens dopamine release, appears to be more pronounced in rats that are conditioned to the testing environment during nicotine pre-treatment (Walter & Kuschinsky 1989, Reid et al 1996, Bevins et al 2001). Pavlovian conditioning is thought to be involved in this process, where rats are placed in a distinct environment reliably paired with nicotine administration (Bevins et al 2005). The context alone (no nicotine during testing) comes to evoke an increase in activity relative to controls, which is thought to reflect a learned association between the context and the psychomotor stimulant effects of nicotine. Walter & Kuschinsky (1989) found evidence for nicotine-conditioned locomotor activity after 6 pairings of a distinct context with nicotine (0.6 mg/kg) administration, as did Reid and colleagues (Reid et al 1996). Bevins and colleagues (Bevins et al 2001) showed a significant conditioning effect after 8 conditioning trials with daily 0.6 and 1.2 mg/kg nicotine (tartrate) injections (highest dose equivalent to ~0.4 mg/kg base). They did not, however, see a significant effect of conditioning in their lowest nicotine treatment group (0.3 mg/kg tartrate).

Smokers have also been observed to develop strong associations between environmental cues associated with smoking and the reinforcing effects of nicotine (Stolerman & Shoaib 1991, Geier et al 2000, Laviolette & van der Kooy 2004). Such smoking-related environmental stimuli might include the sight, smell and taste of cigarettes, a context in which smoking frequently occurred, or other behaviours that frequently accompanied smoking. These environmental cues have been implicated as an important factor in triggering relapse in abstinent tobacco smokers (Balfour et al 2000, Caggiula et al 2001, Liu et al 2006).

As with other psychomotor stimulant drugs, such as cocaine, nicotine administration induces an increase in locomotor activity in rodents, which is enhanced by conditioning to the test environment. Such locomotor conditioning experiments can act as a preclinical model for associative learning processes in human smokers (Bevins et al 2005), and provide a useful starting point for determining the effect of anti-cotinine vaccination on behavioural effects induced by nicotine.

We therefore studied the effects of acute and chronic nicotine administration on locomotor activity, as well as the role of environmental stimuli in the expression of these behavioural effects, and examined the impact of anti-cotinine vaccination on nicotine-induced responses.

Trials 1 – 3 were carried out at Bath University, while Trials 4 & 5 were performed at the University of Newcastle-upon-Tyne (see table in section 2.3.2.3 for details of which experiments were part of which trial).

4.2.2 Methods

4.2.2.1 Apparatus & data collection

Bath apparatus

Spontaneous activity of individual rats was measured using an in-house locomotor activity monitoring system consisting of 24 clear Perspex cages (40 cm (long) x 24 cm (wide) x 20 cm (high)) and cage surrounds containing infrared photocell beam monitors. Any movement of the rats interrupted the photo beams, which was detected and recorded by a PC. Locomotor activity counts were collected at 5 min intervals.

Newcastle apparatus

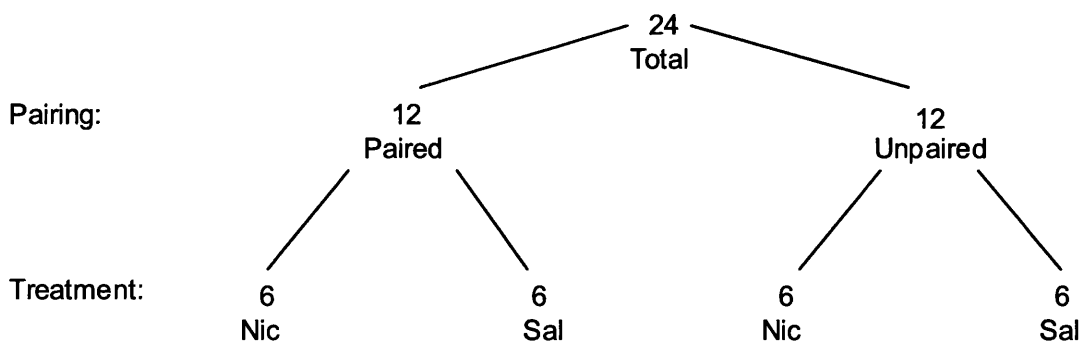
Spontaneous activity of individual rats was measured using infrared photocell beam monitors (Multi-Varimax, Columbus Instruments, Columbus, U.S.) and 4 experimental boxes, with box dimensions of 40 cm (long) x 40 cm (wide) x 30 cm (high). Any movement of the rats interrupted the photo beams, which was detected and recorded by a PC. Locomotor activity counts were collected at 10 min intervals.

4.2.2.2 Conditioned locomotor activity after 4-day nicotine treatment – un-vaccinated pre-trial

Twenty four unvaccinated male Sprague-Dawley rats were used to examine locomotor activity induced by acute administration of nicotine or saline. The locomotor activity cages were also used as conditioning compartments. These experiments were undertaken at Bath University.

Rats were allocated to one of 4 treatment conditions (n = 6 per group): 2 paired groups (nicotine & saline), and 2 unpaired groups (nicotine & saline). During the conditioning phase, rats were injected with either saline or nicotine (0.5 mg/kg, s.c.) and then immediately placed in a locomotor activity cage (paired environment). Control, unconditioned animals received saline or nicotine (0.5 mg/kg, s.c.) in their home cages, 3 h before being placed into the locomotor activity cages (unpaired environment). Locomotor activity was monitored for 45 min and recorded in 5 min time bins. All animals were acclimatised to the locomotor activity room for 30 min before starting the session. This was repeated once a day for 4 days.

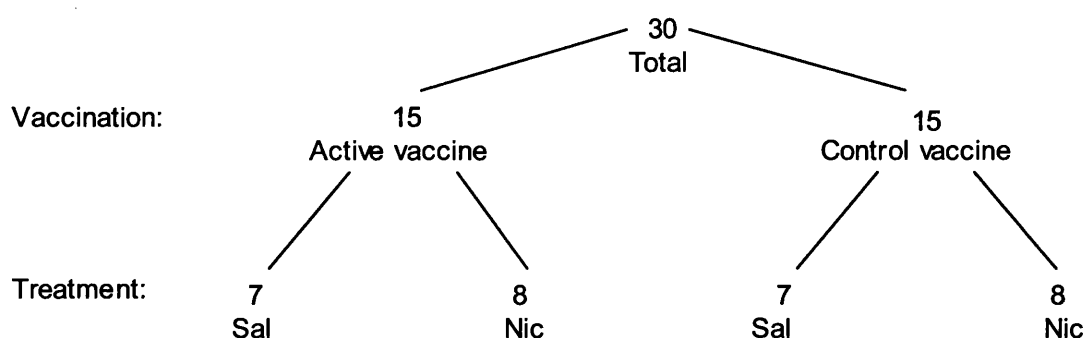
The test phase was conducted as the conditioning phase, except that all rats were given a saline injection, including those that had received nicotine during the conditioning phase. Thus, during the test phase, the locomotor activity was induced by saline or nicotine-associated cues only. All experiments were carried out during the light phase.



4.2.2.3 Conditioned locomotor activity after 4-day nicotine treatment – vaccinated pre-trial

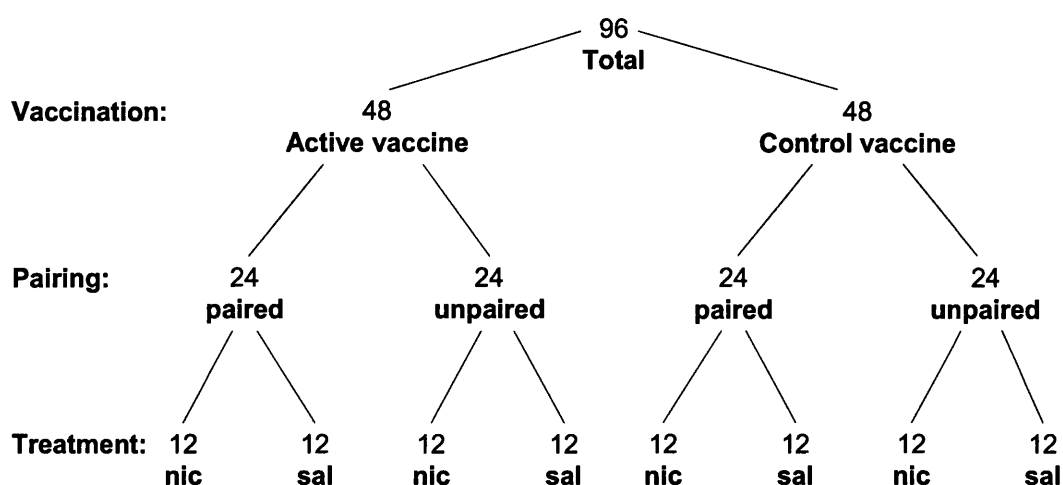
Thirty vaccinated male Sprague-Dawley rats from the adjuvant optimisation experiments (Trial 1, Bath Uni; see section 2.3.2.3.1) were used for this preliminary assessment of the effect of vaccination on locomotor activity induced by acute nicotine administration.

Rats were allocated to one of 4 groups: 2 groups were vaccinated with TT-CotSH, and 2 with TT-Cysteine ($n = 8$ for the nicotine groups, $n = 7$ for the saline groups). Locomotor activity under the paired condition was examined as described in section 4.2.2.2, so all rats received their injections just prior to being placed in the test cages for locomotor activity measurement. All experiments were carried out during the light phase.



4.2.2.4 Conditioned locomotor activity after 4-day nicotine treatment

Ninety six vaccinated male Sprague-Dawley rats (Trial 2, Bath Uni; see section 2.3.2.3.2) were randomised into 8 groups ($n = 12$ per group):



Locomotor activity induced by acute nicotine or saline was examined as described above (see section 4.2.2.2). However, although this set of experiments was also carried out during the rats' light phase, this time the room containing the locomotor equipment was not lit.

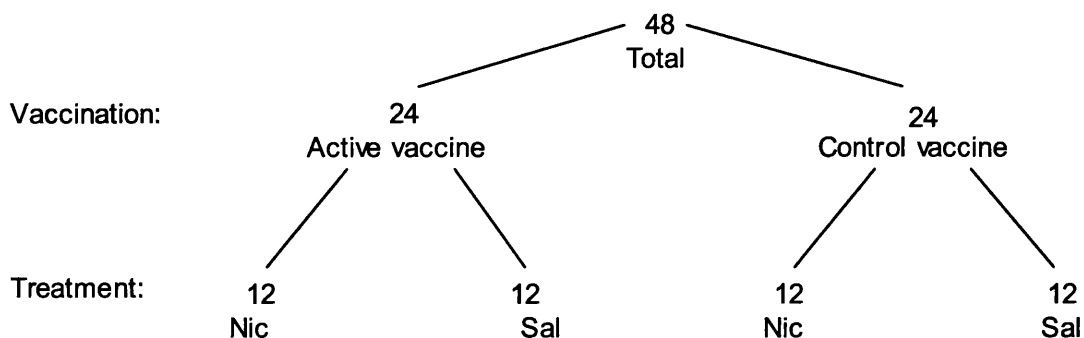
On the final day (test day), results for one run were lost due to the computer crashing whilst recording. This resulted in a group size of $n = 9$ for the test day, instead of $n = 12$.

4.2.2.5 Conditioned locomotor activity over 12-day nicotine treatment

The protocol for these experiments was based on a paper by Kosowski and Liljequist (2005), with minor alterations. Forty eight vaccinated male Sprague-Dawley rats (Trial 3, Bath Uni; section 2.3.2.3.3) were allocated one of 4 treatment conditions ($n = 12$ per group): 2 groups vaccinated with TT-CotSH (saline vs nicotine), and 2 groups vaccinated with TT-Cysteine (saline vs nicotine).

Rats were injected subcutaneously with saline (1 ml/kg) and immediately placed in a locomotor activity cage for 30 min. Rats were then given an injection of either saline or nicotine (0.5 mg/kg, s.c.) and returned to their locomotor activity cage for a further 60 min. Locomotor activity was recorded throughout the 90 min sessions in 5 min time bins. This was done once daily for 12 days.

Unfortunately, data for 4 saline rats could not be obtained due to malfunctioning locomotor cages, resulting in slightly lower numbers for the saline groups ($n = 12$ for nicotine groups, $n = 10$ for saline groups). These rats were however included in the experiment (even if locomotor data was not being generated), in order to retain sample size for subsequent assays (e.g. radioligand binding).



4.2.2.6 Locomotor activity induced by acute nicotine administration

After completion of the conditioned taste aversion experiments at the University of Newcastle (see section 4.4), the 32 vaccinated male hooded Lister rats (16 vaccinated with TT-CotSH, 16 with TT-Cysteine) were housed in their original groups of 4 again, and their locomotor activity in response to acute injections of saline and nicotine examined.

Every second day, rats were injected subcutaneously with saline, 0.1 mg/kg nicotine, or 0.4 mg/kg nicotine, and immediately placed in a locomotor activity box. Drug treatments were administered in a randomised order, with 48 hours between individual treatments to allow any residual drug to be fully metabolised and excreted before the next session. Each animal was tested once in each condition. Locomotor activity was recorded in 10 min time bins for 90 min under each condition.

4.2.2.7 Data analysis

Data are presented as means (\pm S.E.M), and expressed as distance travelled in discrete units per 5 or 10 min intervals, or as the area under the curve (AUC) for a given period of time. Data were analysed using ANOVA (two-way or three-way, depending on the experiment) with repeated measures as needed, and followed by Bonferroni's post hoc analysis.

4.2.3 Results

4.2.3.1 Effects of pairing of drug and environment on locomotor activity after 4-day nicotine treatment – un-vaccinated pre-trial

Initially we studied a small cohort on unvaccinated rats to establish testing procedures and examine effects of treatment, as well as pairing of treatment with the test, on nicotine-induced responses. The results of this pre-trial are illustrated in Figure 4.1. The raw data for each day and the area under the curve for each 45 min session (AUC45) were analysed using a two-way ANOVA (with repeated measures for the raw data), followed by Bonferroni's post-hoc analysis.

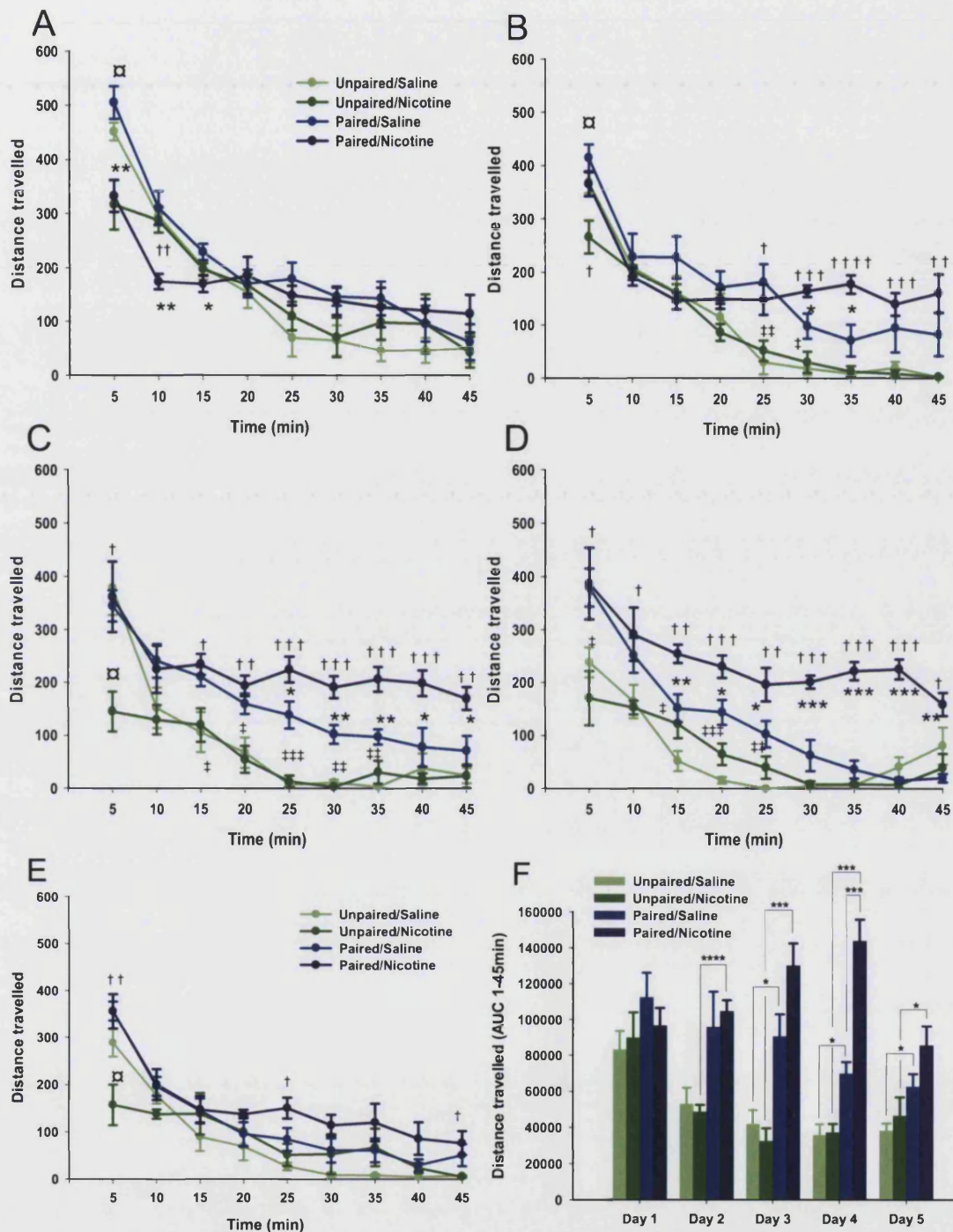
Locomotor activity in all groups decreased gradually over the course of each 45 min session, which was reflected in a significant effect of time ($p < 0.0001$) throughout the 5-day experiment. Both nicotine treatment and pairing of nicotine administration with the test environment resulted in an increase in locomotor activity:

Acute administration of nicotine (0.5 mg/kg, s.c.) initially resulted in a slight reduction of locomotor activity (see Figure 4.1 A), which was more marked in rats whose injections were paired with the locomotor test cage. The analysis for day 1 revealed only effects of time x pairing ($F(\text{time} \times \text{pairing})_{8,160} = 3.074$, $p = 0.0030$) and time x treatment ($F(\text{time} \times \text{treatment})_{8,160} = 7.261$, $p < 0.0001$). Over the following days (see Figure 4.1 B - D), an increase in locomotor activity was observed in the paired nicotine group, which became more pronounced from one day to the next. This effect was even observed, though to a much lesser extent, on day 5 (see Figure 4.1 E), when all groups were injected with saline to test for conditioning effects. The initial increase in activity found in the paired saline group was not as robust or as long lasting as that seen in response to nicotine administration.

These observations were reflected in the results of the statistical analysis: on day 2 (see Figure 4.1 B), a significant main effect of pairing appeared ($F(\text{pairing})_{1,20} = 21.154$, $p = 0.0002$), which persisted until day 5. A significant main effect of pairing x treatment was seen on day 3 ($F(\text{pairing} \times \text{treatment})_{1,20} = 6.686$, $p = 0.0177$; see Figure 4.1 C), which was still present on day 4 (see Figure 4.1 D), when a significant effect of treatment was also observed ($F(\text{treatment})_{1,20} = 18.358$, $p = 0.0004$). Neither of the latter effects was seen on day 5 (see Figure 4.1 E).

Similar results were obtained when examining the area under the curve for each day: there were no significant main effects on day 1, whereas significant main effects of pairing, pairing x treatment, and treatment were observed on days 2 – 5, 3 – 4, and day 4, respectively (see Figure 4.1 F).

Having observed a significant increase in locomotor activity as a result of pairing nicotine injections with the test environment, we studied the effect of anti-cotinine vaccination on nicotine-induced hyperlocomotion.

Figure 4.1: Effects of pairing on nicotine-induced locomotor activity

A-D) Locomotor activity over the 4-day treatment phase; E) Locomotor activity on the test day; Treatment effect (saline vs nicotine) between paired groups: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; Treatment effect (saline vs nicotine) between unpaired groups: $\ast p < 0.05$; Effect of pairing (paired vs unpaired) between nicotine-treated groups: $\dagger p < 0.05$, $\dagger\dagger p < 0.01$, $\dagger\dagger\dagger p < 0.001$; Effect of pairing (paired vs unpaired) between saline-treated groups: $\ddagger p < 0.05$, $\ddagger\dagger p < 0.01$, $\ddagger\dagger\dagger p < 0.001$

*F) Area under the curve for each 45 min session (AUC45); * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$ (Day 5: significant effect of treatment, but not treatment x pairing)*

Unvaccinated rats were administered nicotine (0.5 mg/kg, s.c.) or saline for 4 days, either in the locomotor activity cage (paired) immediately before, or in their homecage (unpaired) 3 h prior to measurement of locomotor activity for 45 min. On day 5 all rats were injected with saline to test for conditioning effects. Movement was detected by means of infrared beams in the cage surround.

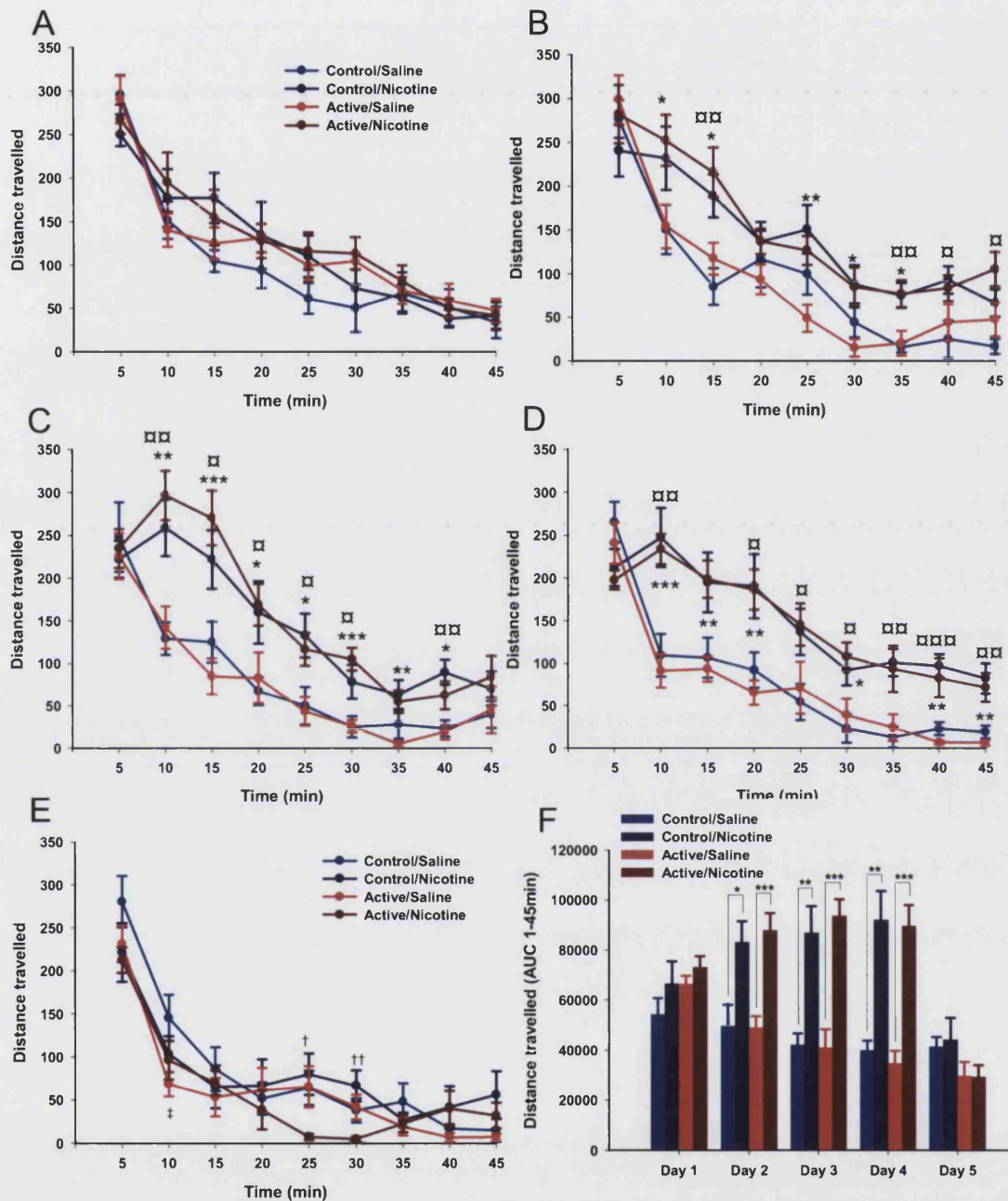
Data presented are mean \pm S.E.M, $n = 6$ per group.

4.2.3.2 Effects of vaccination on locomotor response after 4-day nicotine treatment – vaccinated pre-trial

For the second pre-trial we utilised the remaining vaccinated animals from Trial 1 (see section 2.3.2.3.1), in order to focus on the effects of anti-cotinine vaccination on nicotine-induced locomotor responses. Data analysis was performed as described in section 4.2.3.1, and the results are illustrated in Figure 4.2.

Again, locomotor activity in all groups decreased gradually over the course of each 45 min session (see Figure 4.2 A - D), and the effect of time was highly significant throughout ($p < 0.0001$). Nicotine-induced locomotor hyperactivity was observed from day 2 onwards, with a significant effect of treatment (saline vs nicotine) appearing on day 2 and persisting until day 4 ($p < 0.0001$ throughout). There was no significant effect of treatment x vaccination (see Figure 4.2 A - E), however, on day 5 (see Figure 4.2 E) a significant effect of vaccination (control vs active) alone was observed ($F(\text{vaccination})_{1,26} = 4.935$, $p = 0.0352$).

The analysis of the area under the curve over 45 min yielded similar results (see Figure 4.2 F), with no significant effects on day 1, a significant main effect of treatment ($p < 0.0001$) on days 2 – 4, and a small but significant main effect of vaccination on day 5 ($F(\text{vaccination})_{1,26} = 4.721$, $p = 0.0391$).

Figure 4.2: Effects of vaccination on nicotine-induced locomotor activity

A-D) Locomotor activity over the 4-day treatment phase; E) Locomotor activity on the test day; Treatment effect (saline vs nicotine) between groups immunised with active vaccine: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; Treatment effect (saline vs nicotine) between groups immunised with control vaccine: $\square p < 0.05$, $\square\square p < 0.01$, $\square\square\square p < 0.001$; Effect of vaccination (control vs active) between nicotine-treated groups: $\dagger p < 0.05$, $\dagger\dagger p < 0.01$; Effect of vaccination (control vs active) between saline-treated groups: $\$ p < 0.05$

F) Area under the curve for each 45 min session (AUC45); * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Immunised rats were administered nicotine (0.5 mg/kg, s.c.) or saline for 4 days, in the locomotor activity cage immediately before measurement of locomotor activity for 45 min. On day 5 all rats were injected with saline to test for conditioning effects.

Movement was detected by means of infrared beams in the cage surround.

Data presented are mean \pm S.E.M, $n = 8$ (nicotine) or 7 (saline) per group.

4.2.3.3 Effects of pairing and vaccination on locomotor activity after 4-day nicotine treatment

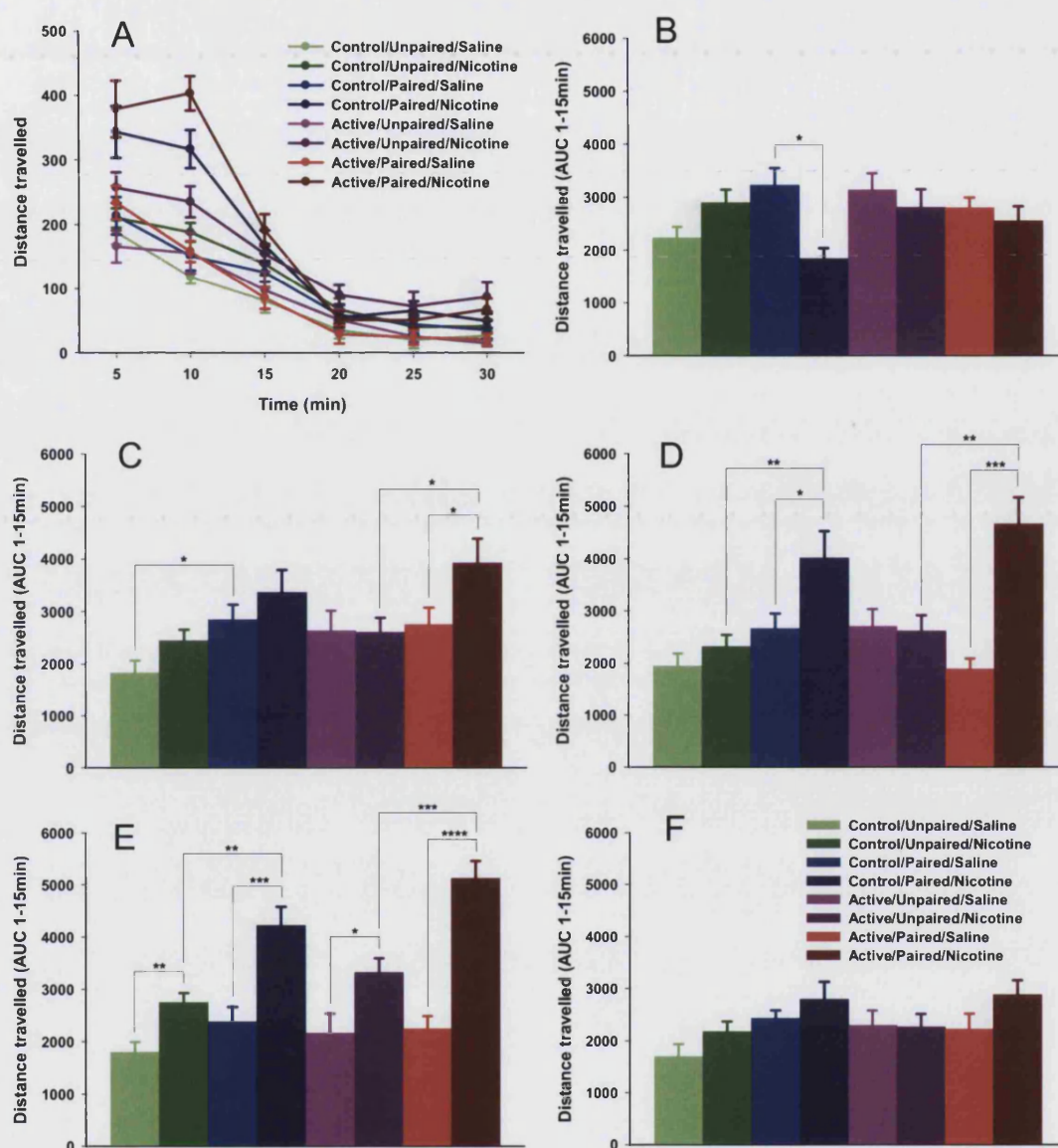
For the full trial we divided the 96 rats, immunised with either TT-Cysteine or TT-CotSH, into 8 groups to examine not just the effect of vaccination against cotinine, but also pairing of the nicotine/saline treatment with the test environment.

Differences between groups were greatest in the first 15 min, so the area under the curve for the first 15 min (AUC15) was analysed by three-way ANOVA, followed by Bonferroni's post-hoc analysis.

Locomotor activity in all groups decreased gradually over the course of each 30 min session (see Figure 4.3 A), and the main effect of time was highly significant throughout ($p < 0.0001$). Both nicotine treatment and pairing of the test environment with nicotine administration resulted in enhanced locomotor activity, whilst vaccination against cotinine displayed a tendency to further increase activity levels:

Examining the AUC15 (see Figure 4.3 B - F), significant interactions of vaccination, treatment and pairing ($F(\text{vaccination} \times \text{treatment} \times \text{pairing})_{1,84} = 7.804$, $p = 0.0065$), and treatment with pairing ($F(\text{treatment} \times \text{pairing})_{1,84} = 6.670$, $p = 0.0115$) were found on day 1. Main effects of treatment ($F(\text{treatment})_{1,84} = 5.616$, $p = 0.0201$) and pairing ($F(\text{pairing})_{1,84} = 12.443$, $p = 0.0007$) appeared on day 2 and persisted until day 4, and a significant interaction of treatment with pairing was also observed on days 3 & 4 ($F(\text{pairing} \times \text{treatment})_{1,84} = 14.365$, $p = 0.0003$ and $F(\text{pairing} \times \text{treatment})_{1,84} = 10.418$, $p = 0.0018$, respectively). The effect of pairing remained significant on day 5 ($F(\text{pairing})_{1,84} = 6.619$, $p = 0.0125$) and the treatment effect was very close to significant ($p = 0.0506$). A three-way interaction on day 3 was also close to significant ($F(\text{vaccination} \times \text{treatment} \times \text{pairing})_{1,84} = 3.852$, $p = 0.0530$), and in addition, a significant main effect of vaccination was observed on day 4 ($F(\text{vaccination})_{1,84} = 4.324$, $p = 0.0406$).

Figure 4.3: Effects of pairing and vaccination on nicotine-induced locomotor activity



A) Sample raw data showing locomotor activity over time (day 4); B-E) Locomotor activity over the 4-day treatment phase; F) Locomotor activity on the test day. Vaccinated rats were administered nicotine (0.5 mg/kg, s.c.) or saline for 4 days, either in the locomotor activity cage (paired) immediately before, or in their homecage (unpaired) 3 h prior to measurement of locomotor activity for 30 min. On day 5 all rats were injected with saline to test for conditioning effects. Movement was detected by means of infrared beams in the cage surround.

Data shown are area under the curve for the first 15 min (AUC15), mean \pm S.E.M, $n = 12$ (A-E) or 9 (F) per group; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

4.2.3.4 Effects of vaccination on locomotor activity over 12-day nicotine treatment

Having looked at the effects of sub-chronic nicotine administration (daily nicotine injections for 4 days, followed by saline on the testing day, see above), we chose to study the effects of longer-term daily injections of nicotine (for 12 days) to assess whether more prolonged exposure to nicotine would result in a greater increase in locomotor activity and a more significant conditioning effect of environmental cues.

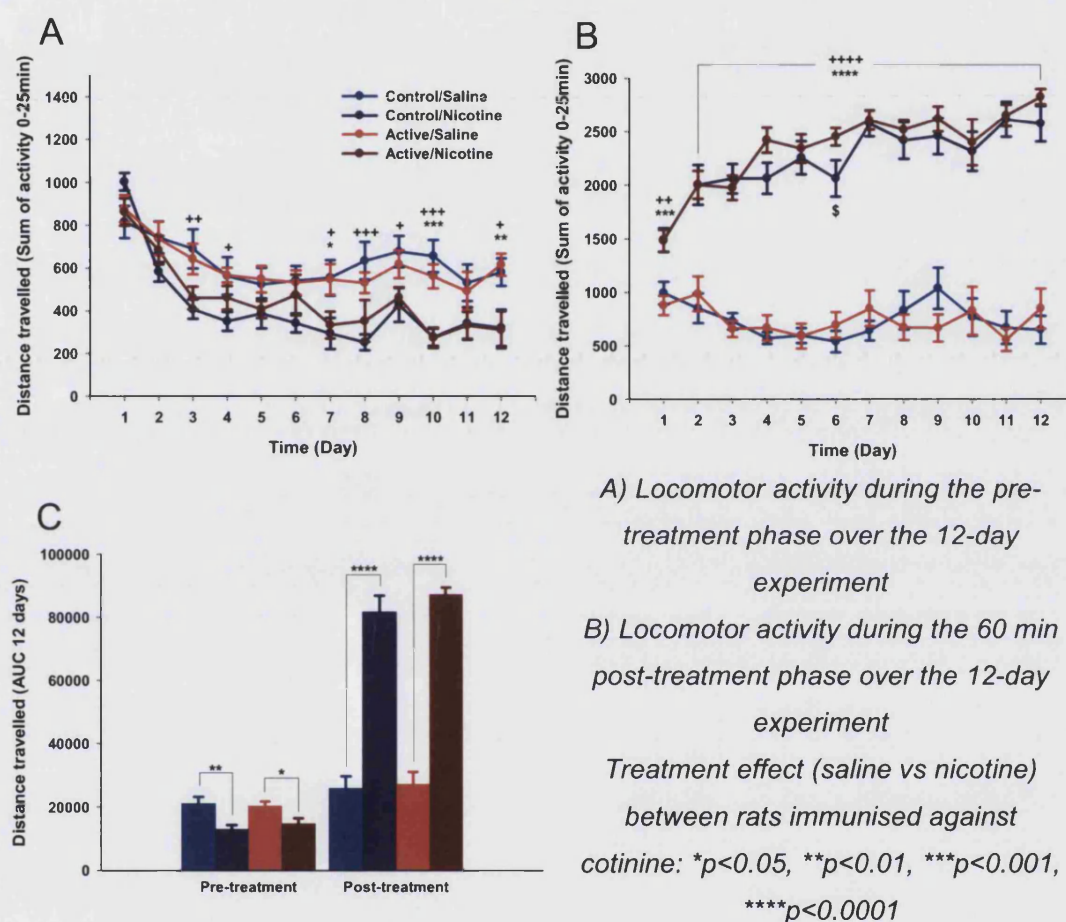
The sum of activity (distance travelled) per day over the 12-day period for the pre-treatment and post-treatment phases is illustrated in Figures 4.4 A and B, respectively, and was analysed using a two-way ANOVA with repeated measures, and Bonferroni's post-hoc test. The area under the curve (AUC) for each group over the 12-day period was also plotted for both pre-treatment and post-treatment phases (see Figure 4.4 C), and analysed by two-way ANOVA and Bonferroni's post-hoc analysis.

Each day, the locomotor activity in all groups gradually declined over the course of the pre-treatment phase (data not shown). A reduction in locomotor activity levels was also observed from one day to the next until a plateau was reached around day 3 (see Figure 4.4 A). This effect was greater in the groups receiving nicotine during the treatment phase, than in those receiving saline ($F(\text{treatment})_{1,40} = 14.508$, $p = 0.0005$). Examination of the area under the curve for each group (see Figure 4.4 C) revealed a significant effect of drug treatment ($F(\text{drug})_{1,40} = 17.858$, $p = 0.0001$). The effect of time was also highly significant throughout ($p < 0.0001$).

Over the course of the post-treatment phase, locomotor activity in the groups injected with saline again decreased over time (each day), whilst in the groups that had received nicotine injections, activity increased over the initial 15 min post-injection until a plateau was reached, usually around 20 – 30 min (data not shown). Levels of overall activity in the saline-treated groups remained relatively constant over the 12-day experiment, whereas activity of nicotine-treated groups gradually increased from day to day (see Figure 4.4 B). This resulted in a highly significant main effect of treatment ($F(\text{treatment})_{1,40} = 227.637$, $p < 0.0001$) over the 12 day period, which was repeated in the comparison of AUC

($F(\text{treatment})_{1,40} = 217.461$, $p < 0.0001$; see Figure 4.4 C). The effect of time was also highly significant throughout ($p < 0.0001$).

Figure 4.4: Effect of vaccination on locomotor activity over 12-day nicotine treatment



A) Locomotor activity during the pre-treatment phase over the 12-day experiment

B) Locomotor activity during the 60 min post-treatment phase over the 12-day experiment

Treatment effect (saline vs nicotine) between rats immunised against cotinine: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

Treatment effect (saline vs nicotine) between rats immunised with TT-Cysteine: + $p < 0.05$, ++ $p < 0.01$, +++ $p < 0.001$, ++++ $p < 0.0001$; Vaccination effect (active vs control) between nicotine-treated rats: \$ $p < 0.05$

C) Area under the curve (AUC) for A & B; * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$

Vaccinated rats were injected with saline (1 ml/kg, s.c.) and placed in a locomotor activity cage for 30 min (pre-treatment phase). They were then given another injection of either nicotine (0.5 mg/kg, s.c.) or saline, and locomotor activity measured for a further 60 min (post-treatment phase). This procedure was repeated for 12 days.

Movement was detected by means of infrared beams in the cage surround.

Data presented are mean \pm S.E.M, $n = 12$ (nicotine) or 10 (saline) per group.

Vaccination against cotinine tended to further increase nicotine-induced locomotor activity; however, a significant effect of vaccination was only found (post-treatment) on day 6 ($F(\text{vaccination})_{1,40} = 4.703$, $p = 0.0361$) and a trend towards a vaccination effect was also observed on day 4 post-treatment ($F(\text{vaccination})_{1,40} = 3.637$, $p = 0.0637$). In addition, a significant interaction between time and vaccination was seen post-treatment on day 4 ($F(\text{time} \times \text{vaccination})_{11,440} = 2.058$, $p = 0.0221$), and on day 12 (post-treatment) the 3-way interaction between time, vaccination and treatment was also very nearly significant ($F(\text{time} \times \text{vaccination} \times \text{treatment})_{11,440} = 1.809$, $p = 0.0503$).

4.2.3.5 Effects of vaccination on locomotor activity induced by acute nicotine administration

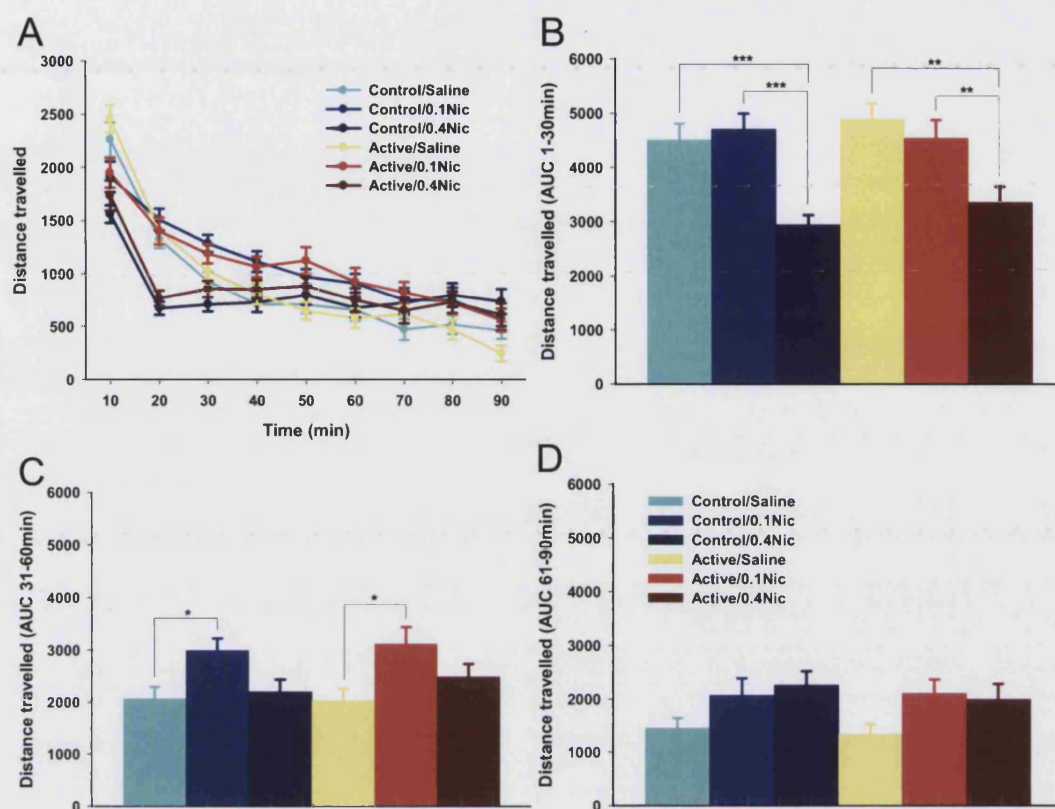
To complement the experiments looking at the effects of chronic and sub-chronic nicotine exposure (see above), the effects of vaccination on the acute response to saline and nicotine administration (0.1 & 0.4 mg/kg, s.c.) were examined over a 90 min period.

The raw data for each day were analysed using a two-way ANOVA with repeated measures, followed by Bonferroni's post-hoc analysis. In addition, the area under the curve for 1 - 30, 31 - 60, and 61 - 90 min (AUC30, AUC60 and AUC90, respectively) were also compared by two-way ANOVA with Bonferroni's post-hoc tests.

A main effect of treatment ($F(\text{treatment})_{2,90} = 5.109$, $p = 0.0079$) was seen when examining the raw data (see Figure 4.5 A), along with a highly significant effect of time ($p < 0.0001$). The treatment effect was also apparent upon analysis of the AUC data (see Figure 4.5 B - D), but decreased in significance over time ($F(\text{treatment})_{2,90} = 24.974$, $p < 0.0001$, $F(\text{treatment})_{2,90} = 8.446$, $p = 0.0004$ and $F(\text{treatment})_{2,90} = 5.828$, $p = 0.0042$, for AUC30, AUC60 and AUC90, respectively).

Administration of the higher nicotine dose (0.4 mg/kg) produced an initial depression, followed by an increase in locomotor activity, whereas the lower dose (0.1 mg/kg) had no initial depressant effect on activity levels and resulted in a more rapid increase in locomotor activity. No significant effects of vaccination were observed during these experiments.

Figure 4.5: Effect of vaccination on locomotor activity induced by acute nicotine administration



A) Locomotor activity over the 90 min measurement period; B) Area under the curve for the first 30 min (AUC30); C) Area under the curve for 31-60 min (AUC60); D) Area under the curve for 61-90 min (AUC90)

Immunised rats were injected with nicotine (0.4 or 0.1 mg/kg, s.c.) or saline, and locomotor activity recorded for 90 min. Movement was detected by means of infrared beams in the cage surround.

Data presented are mean \pm S.E.M, $n = 16$ per group; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

4.2.4 Discussion

In this section we examined nicotine-induced changes in locomotor activity in rats, using a variety of nicotine treatment regimes, as well as studying the effects of conditioning to the test environment on these responses.

Effects of short intermittent nicotine exposure on locomotor activity

Initially we utilised a 5-day treatment schedule, consisting of 4 days of nicotine or saline treatment, followed by a test day for conditioning effects. Using this

treatment schedule we observed a progressive increase in nicotine-induced hyperlocomotion over the 4-day treatment period, consistent with previous findings (Benwell & Balfour 1992, Reid et al 1996, Bevins et al 2001). This enhancement of locomotor activity was more significant and was observed earlier in rats whose nicotine administration was paired with the test environment (see Figures 4.1 & 4.3), which is in agreement with other studies (Reid et al 1996, Bevins et al 2001).

In addition, the nicotine-induced hyperlocomotion was more pronounced and appeared sooner in the paired group vaccinated against cotinine, than in the paired group vaccinated with TT-Cysteine (see Figure 4.3). Together with the significant 3-way interaction on day 3 and the vaccination effect on day 4, this was an indication that the anti-cotinine vaccine might be increasing nicotine-induced locomotor activity.

There are a number of possible explanations for the absence of significant effects on the test day of the full experiment (see section 4.2.3.3). It is possible that the anti-cotinine antibody titres in the vaccinated groups were too low to have had a noticeable effect on nicotine-induced behaviour. This is especially likely in the vaccinated pre-trial (see section 4.2.3.2), as rats in this experiment ("Adjuvant X/Y/Z" groups) had anti-cotinine antibody titres of around 1:2000 (see section 2.3.3.3 & Figure 2.4), whereas in the full experiment (see section 4.2.3.3), where a trend towards a vaccine effect was detected, titres were closer to 1:7500 (see Figure 2.10 & section 2.3.3.3).

Another possibility is that the stimuli associated with the test environment may not have been sufficient to produce a conditioned response, although the fact that we detected a significant pairing effect in our first pre-trial (see section 4.2.3.1 & Figure 4.1) suggests this was not the case. Hakan and Ksir (1985) observed that even after 9 days of nicotine administration (0.2 mg/kg) associated with visual and auditory cues, a challenge with saline and cues alone on day 10 produced locomotor activity levels very similar to saline pre-treated rats and significantly lower than the response to nicotine and cues on the previous and the following day. Bevins and colleagues (Bevins et al 2001) on the other hand, showed a significant conditioning effect after 8 conditioning trials with daily 0.6 and 1.2 mg/kg nicotine (bitartrate) injections. They did not, however, see a significant effect of conditioning in their lowest nicotine

treatment group (0.3 mg/kg), so it is possible that the lack of conditioning observed by Hakan and Ksir could have been due to an insufficient dose. This is less likely to be the case for our studies, as our dose of 0.5 mg/kg (base) is not that dissimilar to the 1.2 mg/kg nicotine tartrate dose (equivalent to ~0.4 mg/kg nicotine base) used by Bevins (Bevins et al 2001).

Finally, the 4-day nicotine pre-treatment might not have been long enough to produce a measurable conditioning effect on locomotor activity in the absence of nicotine on day 5. Bevins et al (2001) used an 8-trial conditioning procedure, observing a significant conditioning effect on day 9. However, conditioning effects have also previously been observed after only 5 days of nicotine pretreatment (Ksir et al 1987, Walter & Kuschinsky 1989, Reid et al 1996).

The choice of a 4-day pre-treatment experimental design was based on advice from Christian Heidbreder (GSK), whose group had used this schedule routinely and observed reliable nicotine-induced hyperlocomotion on the test day (personal communication). Such a difference between laboratories in terms of results might have been due to a number of factors, such as rat strain (Sprague-Dawleys vs Lister Hooded), housing conditions (group vs single), or the size of the locomotor activity boxes. The rats used by the GSK group were also significantly younger and smaller/lighter than ours, as they did not undergo ~3 months of vaccinations prior to the experiment, which might also have impacted on their locomotor activity responses.

Effects of 12-day intermittent nicotine exposure on locomotor activity

In order to address the possibility that the lack of conditioned hyperlocomotion on day 5 might have been due to the relatively short nicotine treatment period, we looked at the effects of a 12-day treatment schedule on nicotine-induced locomotor activity. Rather than using a longer version of our previous schedule, we chose a paradigm used by Kosowski and Liljequist (2005) because it allowed us to look at conditioning effects as well as nicotine effects in the same subjects. This meant we could use a simpler experimental design requiring fewer groups, and therefore fewer animals. To control for injection effects, we modified the design slightly, to include a saline injection at the beginning of the 30 min pre-treatment phase (as suggested by Kosowski & Liljequist in their discussion).

We observed that repeated pairing of nicotine (0.5 mg/ kg, s.c.) with the testing environment elicited nicotine-conditioned hypolocomotion in the pre-treatment phase over the 12-day period (see Figure 4.4), in contrast with the increase in locomotor activity observed by Kosowski & Liljequist (2005). The progressive increase in locomotor activity observed in response to nicotine administration was, however, consistent with the results obtained by Kosowski and Liljequist, as well as previous studies (Benwell & Balfour 1992, Reid et al 1996), and was highly significant over the entire 12-day period. Nisell and colleagues have also shown a substantial increase in nicotine-induced hyperlocomotion, as well as an enhancement of DA release from the PFC and NAcc, in rats pretreated with nicotine, using the same dose of nicotine (0.5 mg/kg) and length of treatment (12 days; Nisell et al 1996).

There was a general tendency towards slightly higher activity levels (post-treatment) in the group vaccinated against cotinine, compared to controls, however this was not significant. Again this relatively slight effect may have been due to low anti-cotinine antibody titres, since those achieved in this Trial (~1:3,000) were approximately half as high as those obtained in the previous Trial (~1:7,500; see Figure 2.10 & section 2.3.3.3).

Effects of acute nicotine administration on locomotor activity

Finally, we examined the acute effects of different doses of nicotine on the locomotor activity of rats over a 90 min period. Administration of 0.4 mg/kg nicotine resulted in an initial depressant effect on locomotor activity, followed by an increase, which is consistent with previous studies (Clarke & Kumar 1983, Walter & Kuschinsky 1989, Bevins et al 2001, Bevins & Palmatier 2003). The 0.1 mg/kg dose had no initial depressant effect on activity levels, and resulted in a more rapid increase in locomotor activity. Bevins et al (2001) observed similar dose-dependent effects, with a decrease in locomotor activity after administration of 1.2 mg/kg (bitartrate; equivalent to ~0.4 mg/kg base), but not 0.3 or 0.6 mg/kg.

We did not observe any effects of vaccination, which is likely to be due to the low titres (~1:800) achieved in this Trial (see Figure 2.10 & section 2.3.3.3). This was a result of the mislabelling of vials containing a fresh batch of conjugates

for vaccination (see section 2.3.2.3.4) and the consequent vaccination of control animals with active vaccine and vice-versa (see section 2.3.2.3.6).

Effects of immunisation on locomotor activity

Passive immunisation against nicotine has been shown to block the locomotor stimulant effect of an acute dose (0.28 mg/kg, s.c.) of nicotine (Pentel et al 2000). This is thought to be the result of nicotine-specific antibodies binding the drug molecules and preventing them from crossing the blood-brain-barrier and acting on nAChRs. Furthermore, both vaccination and passive immunisation have been shown to attenuate the locomotor stimulant effects of nicotine in rats after repeated exposure to nicotine and, in the case of passive immunisation, in a dose-dependent fashion (Carrera et al 2004).

According to the premise for the cotinine vaccine approach, vaccination of rats against cotinine would be expected to enhance nicotine-induced stimulation of locomotor activity, due to cotinine-specific antibodies retaining cotinine in the bloodstream and preventing it from antagonising the effects of nicotine.

However, we did not detect any robust effects of vaccination against cotinine on locomotor activity levels. This may be a result of the relatively low anti-cotinine antibody titres generally achieved in the vaccinated rats. On the other hand, some encouraging signs of a vaccination effect were observed in the locomotor experiments conducted as part of Trial 2, in which the highest anti-cotinine antibody titres were obtained (see Figure 2.10 & section 2.3.3.3). In this study there was evidence of a possible enhancement of nicotine-induced locomotor activity as a consequence of vaccination against cotinine (see section 4.2.3.3); however, no clear correlation between antibody titre and locomotor activity (on day 4) was found (data not shown), possibly due to the large variability in terms of antibody levels within each group. If the number of subjects per group had been greater and higher antibody titres had been achieved, this might have resulted in a more significant impact on locomotor activity levels.

4.3 Effects of anti-cotinine vaccination on intravenous self-administration of nicotine

4.3.1 Introduction

In smokers, nicotine self-administration appears to be motivated by both positive and negative reinforcement. Negative reinforcement refers to the relief of nicotine withdrawal symptoms, including nervousness, restlessness, irritability, anxiety, and impaired concentration and cognitive function, whereas positive reinforcing effects include relaxation, reduced stress, enhanced vigilance, and improved cognitive function and mood. Humans have been shown to like and self-administer oral, nasal, and intravenous nicotine, and the associated reinforcing effects and self-administration behaviour can be blocked by pretreatment with the nicotinic antagonist mecamylamine (Benowitz 1996, Rose & Corrigall 1997, Caggiula et al 2001).

Self-administration studies have shown that many drugs considered to be addictive in humans can serve as positive reinforcers for laboratory animals, including amphetamine, cocaine, morphine, heroin, phencyclidine and nicotine (Stolerman & Shoaib 1991). Just as a large portion of the human population is drawn to tobacco use on a daily basis, rodents will self-administer nicotine (i.v.) when the opportunity is presented in the laboratory (Rose & Corrigall 1997, Caggiula et al 2001, Mansvelder & McGehee 2002). One of the primary advantages of the self-administration model is its resemblance to real-life drug-taking behaviour in humans, both in terms of the repeated intake of small doses of nicotine, but also in the voluntary nature of the nicotine administration.

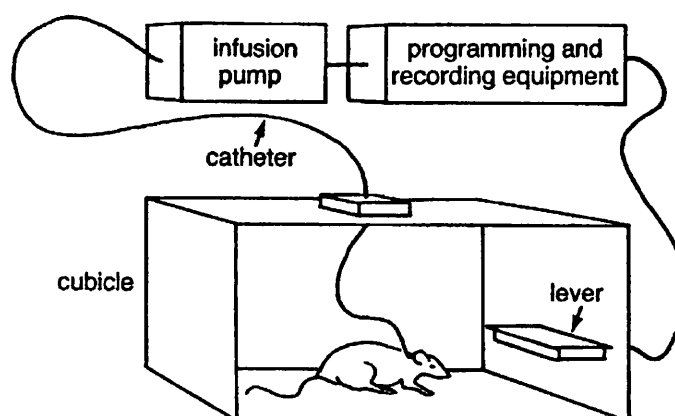
In rodent self-administration studies (see Figure 4.6), the drug is obtained through a chronically indwelling catheter (implanted intravenously during brief surgical anaesthesia), and serves to reinforce behaviour (usually lever-pressing or nose-poking). Access to the drug is generally restricted so that animals must respond a certain number of times or within a certain time in order to receive a drug infusion. A time-out period after each infusion serves to minimise the risk of adverse and toxic effects, such as seizures, as a result of cumulative actions of repeated doses (Rose & Corrigall 1997).

Animal self-administration models can differ in several ways, such as whether the schedule of access to the drug is continuous or limited and intermittent, whether animals are maintained on free feeding or restricted feeding schedules,

and whether animals receive prior operant training and/or drug exposure before the first self-administration session. Reliable self-administration of nicotine has been reported under all of the above conditions. Limited, intermittent access (such as 1 - 2 h per day) appears to result in more rapid acquisition and higher, more stable rates of drug-maintained behaviour (Goldberg et al 1983, Henningfield & Goldberg 1983). On the other hand, continuous access is generally a better model for chronic, continuous drug exposure or patterns of administration (Donny et al 1998).

Figure 4.6: Intravenous self-administration apparatus

The rat is connected to an infusion pump via an intravenous cannula. Presses on the active lever result in an infusion of nicotine. A second lever (not pictured) is inactive, ie responses on that lever are recorded but have no programmed consequence. (from Stolerman 1992)

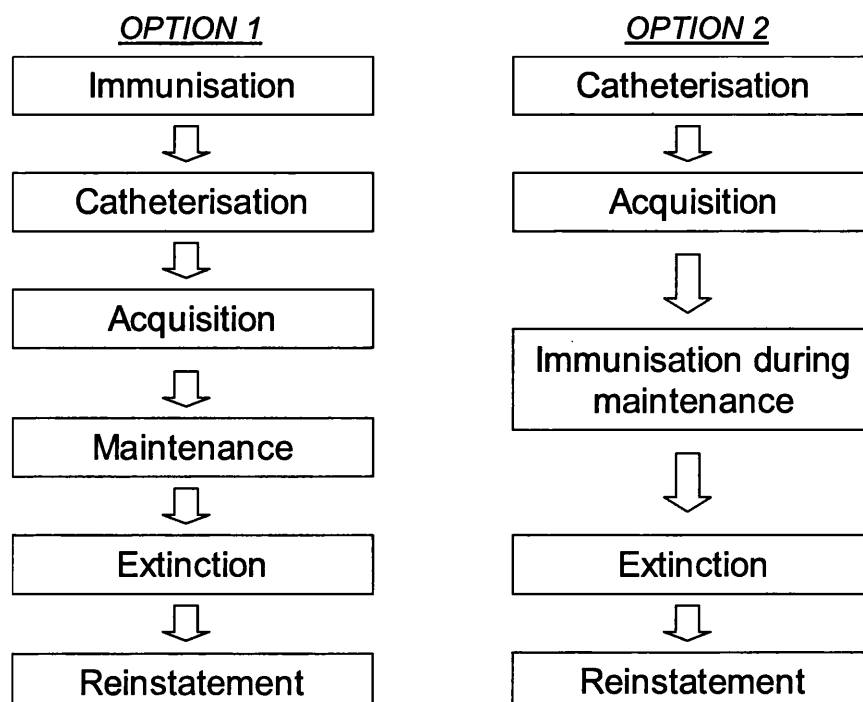


Self-administration behaviour in rats can be extinguished by substituting saline for nicotine, or by pre-treatment of animals with the nicotinic antagonists mecamylamine or DH β E by subcutaneous injection (Corrigall & Coen 1989, Shoaib et al 1997, Watkins et al 1999). Infusion of DH β E into the VTA prior to the start of the nicotine self-administration session also results in a significant decrease in the number of nicotine infusions taken, but does not affect responding for food or cocaine. However, the same dose infused into the NAcc is without effect on nicotine self-administration (Corrigall et al 1994). Furthermore, nicotine self-administration is attenuated in β 2 knockout mice, providing additional evidence for the involvement of β 2* nAChRs in nicotine reinforcement (Picciotto et al 1998). A marked reduction in self-administration of nicotine can also be observed when the mesolimbic DA system is lesioned by injecting 6-OHDA into the NAcc (Corrigall et al 1992), but also when rats are administered selective D1 and D2 dopamine receptor antagonists (Corrigall &

Coen 1991), suggesting that DA transmission plays a key role in mediating nicotine self-administration behaviour. Exposing rats to nicotine prior to experimental sessions (either by injections or by chronic subcutaneous infusion via an osmotic minipump) has also been shown to dose-dependently reduce responding (Corrigall & Coen 1989, LeSage et al 2002).

Although somewhat invasive, intravenous self-administration techniques have the advantage of eliminating the influence of non-pharmacological factors, such as the taste of drug solutions, on drug intake. Also, there is only a very small delay between lever-pressing responses and delivery of the drug (i.e. immediate reward), which facilitates learning of the required behaviour. Drug-taking behaviour can be sustained over long periods of time, and dose-response as well as control data can be obtained from the same subject. In addition, various parameters of addiction can be studied, from acquisition and maintenance of drug-taking behaviour to withdrawal effects and reinstatement by nicotine and/or associated cues. Self-administration techniques are therefore considered to be one of the most direct and best-established methods for assessing the positively reinforcing effects of addictive drugs (Stolerman & Shoaib 1991). For this reason, we chose to examine the effects of vaccination against cotinine on intravenous self-administration of nicotine in rats.

Initially we discussed two schedule options for our experiments:



With option 1, rats would not be implanted with a catheter until the vaccination schedule had been completed. This would allow for monitoring of titres, and possible exclusion of rats with a low immune response prior to catheterisation. Although with this timeline it would be possible to examine the effects of vaccination on the acquisition of self-administration, this would not accurately reflect the proposed application in smokers, where vaccination would be used to support cessation.

Option 2 on the other hand, mirrors a smoker's cessation attempt more closely, as vaccinations would not take place until self-administration behaviour had been established. The main drawback with this option is the length of time the intravenous catheters would have to remain patent (~5 - 6 months) in order to complete all phases of the experiment.

After considering both options, and bearing in mind that the vaccine was designed to assist with smoking cessation, we decided to pursue option 2, as it mimicked the human situation (smokers attempting to quit) more closely, and would potentially enable us to examine the effects of anti-cotinine vaccination on the maintenance, extinction, and reinstatement of nicotine self-administration.

4.3.2 Methods

4.3.2.1 Animals

Male hooded Lister rats (Harlan UK) initially weighing 200 - 250 g were acclimatised to the animal house for approximately one week before surgery and had access to food and water *ad libitum*.

4.3.2.2 Apparatus

Standard operant chambers (Med-Associates, Vt., USA), consisting of a Plexiglas enclosure with one house light, two levers, one tether and fluid swivel, were used. One lever was defined as active and presses on this lever resulted in fluid infusions and extinction of the house light for 20 s; presses on the other lever were recorded but had no programmed consequences. Catheters were connected to an infusion pump (Razal; MED-Associates, Ind., USA) with a syringe containing nicotine solution (saline during extinction and reinstatement).

The operant chambers were controlled by computer, using the MED-Associates MEC-PC software package.

4.3.2.3 Surgery and recovery

Under surgical anaesthesia (mixture of medetomidine 0.3 mg/kg and ketamine 70 mg/kg, i.p.), a chronic Silastic catheter was implanted into the external jugular vein and tunnelled subcutaneously by Dr Shoaib as described previously (Shoaib et al 1997). The catheter was then externalised via a head mount (by me). Daily flushing with 0.9 % physiological saline containing Baytril (enrofloxacin; 0.16 mg/kg per day) and heparin (30U/ml) maintained the patency of the intravenous catheter.

Rats were housed individually post-operatively until they had regained their pre-operative weights. They were then housed in pairs for the duration of the self-administration experiments.

4.3.2.4 Acquisition

In 1 h limited access sessions, rats were given the opportunity to lever-press for intravenous infusions of nicotine (0.03 mg/kg per infusion) as described previously (Shoaib et al 1997). A stimulus light was utilised to signal the availability of nicotine, which was turned off for 20 s during the time-out period after an infusion. Initially each press on the active lever resulted in a nicotine infusion (fixed ratio 1, FR-1). Once rats showed response accuracy with at least 80 % of the responses on the active lever and with stable intake of nicotine (± 2 infusions) over 2 days, the number of responses required to produce an infusion was increased progressively to 2 and finally 3 lever presses (FR-3).

4.3.2.5 Vaccination during stable maintenance

Vaccinations began once a rat exhibited stable levels of nicotine self-administration under a FR-3 schedule of reinforcement (less than 30 % variability from the mean number of infusions self-administered over three sessions). Animals were vaccinated with TT-CotSH or TT-Cysteine according to the schedule described in Chapter 2 (see section 2.2.3.4). Self-administration sessions continued throughout the vaccination period, except for the vaccination days. Upon completing the course of vaccinations, rats were again

required to reach stable levels of nicotine self-administration before progressing to the extinction phase.

4.3.2.6 Extinction

During the extinction phase of the experiment, presses on the active lever were recorded, but had no programmed consequences. As well as removing the nicotine infusion (infusion lines were connected to a saline-filled syringe), the cues predicting the onset of nicotine infusion were also eliminated: infusion pumps were turned off, and the stimulus light was left on for the entire session (time-out period not used).

4.3.2.7 Reinstatement tests

Once lever-pressing behaviour had been extinguished (3 days of $\leq 20\%$ of stable maintenance baseline), rats were challenged with systemically administered nicotine (0.05, 0.1, or 0.2 mg/kg) and no cues, or saline (s.c.) plus cues associated with pressing the active lever (pump noise on, stimulus light switching off), in a randomised order. Active lever presses were recorded throughout; they either had no programmed consequences (nicotine test), or triggered cues (see above; saline test). After each challenge, rats were again required to reach extinction criteria prior to the next reinstatement test.

4.3.2.8 Data Analysis

During the maintenance phase, responses over the 10 sessions following each vaccination were compared using ANOVA with repeated measures, followed by Bonferroni's post-hoc test where required. Response rates on the active lever on reinstatement test days were compared to the mean response levels over the preceding 3 extinction days (baseline) by means of unpaired t-tests.

4.3.3 Results

4.3.3.1 Surgery and recovery

Surgery was performed on a total of 58 male hooded Lister rats. Recovery rates were generally good, with most rats regaining their pre-operative weight within a week of surgery, and infection as a result of surgery was rare. Of an initial 37 animals that were catheterised, only one was culled because of infection post-

surgery. In order to replace rats excluded over the course of the acquisition period due to blocked catheters and/or lack of acquisition of self-administration behaviour, a further 21 animals were implanted with catheters (2 were culled post-surgery due to a blocked catheter and infection, respectively). In all, a total of 55 rats proceeded to the acquisition phase.

4.3.3.2 Acquisition

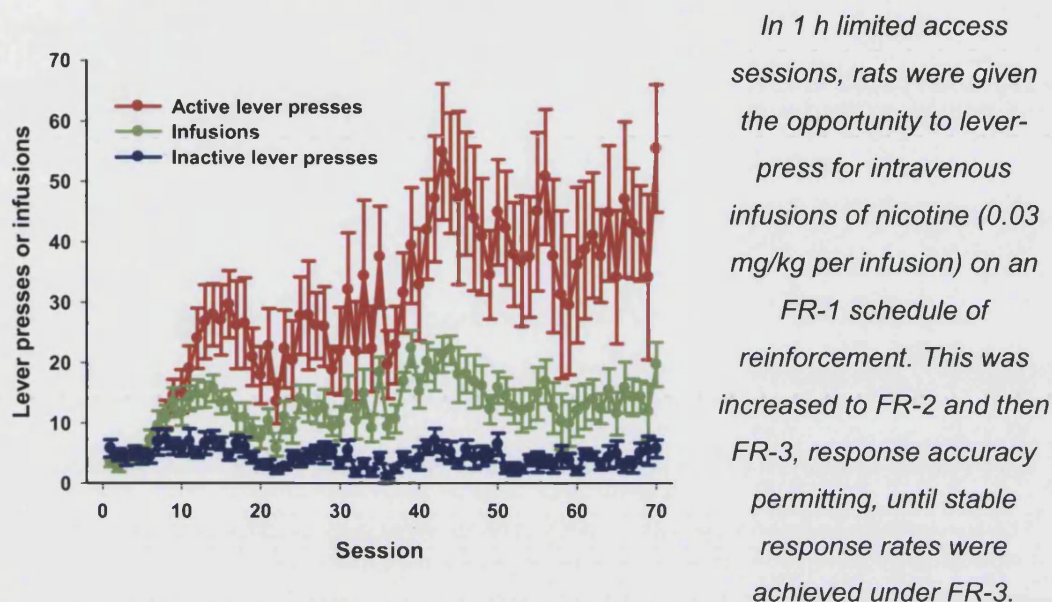
Acquisition of nicotine self-administration behaviour is summarised in Figure 4.7. Response rates on the inactive lever were very low throughout the experiments. Initially acquisition rates were good, and some rats progressed from FR1 to FR2 and then FR3 relatively quickly. However, levels of self-administration behaviour were soon found to be inconsistent from session to session in the majority of animals. In an attempt to stabilise responding rates, rats were switched from daily sessions to sessions every second day (approximately around session 40). This appeared to improve rates of responding to a certain extent; however it also meant that data acquisition was very much slower.

As other studies have only measured self-administration behaviour for 2 – 6 weeks (Shoaib et al 1997 & 2003, Donny et al 1998, Caggiula et al 2001, Liu et al 2006), and we were unsure how long the catheters were likely to remain patent, we switched back to daily sessions, in the hope that rats that had acquired self-administration behaviour would be able to maintain it with more frequent sessions. Several animals that had satisfied stability criteria were able to progress to the next stage of the experiment. The remaining rats still showed highly variable rates of lever pressing, and most were therefore not able to move on to the vaccination phase.

The variability of the response rate resulted in a high rate of attrition, and consequently, of the 55 rats that entered the experiment, only 18 satisfied the criteria for stable maintenance. Figure 4.7 only shows the data for these 18 animals. Some rats acquired the nicotine self-administration behaviour a lot faster than others, and progressed to the next phase of the experiment whenever they had reached stability. The graph shows the first 70 sessions, during which most of the 18 rats satisfied stability criteria; in the interest of clarity, the data for the additional sessions for the remaining animals is not

depicted, as the decreasing sample number resulted in greatly increased error bars. (The last rat to complete acquisition and move into the vaccination phase required ~60 sessions in addition to those shown in Figure 4.7.)

Figure 4.7: Acquisition of nicotine self-administration behaviour



Data shown are mean \pm S.E.M for the 18 animals that completed the acquisition phase (n decreases over time) for the first 70 sessions (see section 4.3.3.2).

4.3.3.3 Effects of vaccination on stable maintenance

Rats that had satisfied criteria for stable maintenance (see section 4.3.2.4) were given 5 vaccinations of TT-CotSH or TT-Cysteine over a ~3-month period indicated by black arrows in Figures 4.9 A – C. The grey arrow indicates the start of the extinction period. In the interest of clarity, mean responses during the first 10 sessions after each vaccination are shown; rats completed varying numbers of sessions during the inter-vaccination periods, which resulted in large error bars towards the end of each period.

Lever-pressing response rates during the vaccination phase remained rather variable between sessions (see Figure 4.9 A), although the rats vaccinated against cotinine generally had higher response levels on the active lever (and therefore more infusions (see Figure 4.9 B)), than those vaccinated with control vaccine. There was a significant effect of vaccination (see Figure 4.8) on active lever presses and infusions over the 10 sessions following the 4th ($F(\text{vaccination})_{1,113} = 15.450$, $p=0.0001$ and $F(\text{vaccination})_{1,113} = 15.661$,

$p=0.0001$) and 5th immunisations ($F(\text{vaccination})_{1,117} 28.117$, $p<0.0001$ and $F(\text{vaccination})_{1,117} 28.388$, $p<0.0001$). However vaccinated rats also showed elevated levels of responding on the inactive lever (see Figure 4.8 & 4.9 C), compared to control vaccinated rats, indicative of a general increase in activity.

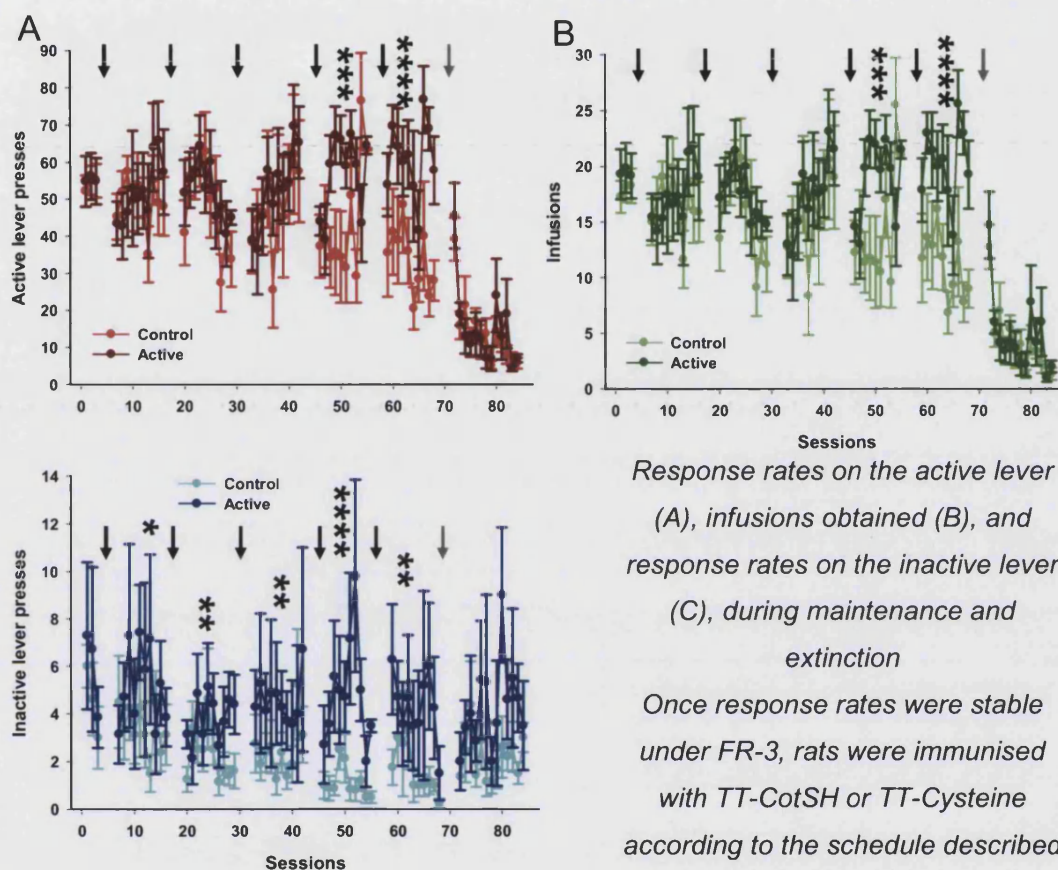
Figure 4.8: Significant effects of vaccination on maintenance of nicotine self-administration

| | After vaccination No. | Sessions |
|-----------------------|--|------------------------|
| Active Lever | 4 th : $F(\text{vacc})_{1,113} = 15.450$ ($p = 0.0001$) | 3-5 & 8 ($p < 0.05$) |
| | 5 th : $F(\text{vacc})_{1,117} = 28.117$ ($p < 0.0001$) | 9 ($p < 0.01$) |
| Infusions | 4 th : $F(\text{vacc})_{1,113} = 15.661$ ($p = 0.0001$) | 3-5 & 8 ($p < 0.05$) |
| | 5 th : $F(\text{vacc})_{1,117} = 28.388$ ($p < 0.0001$) | 9 ($p < 0.01$) |
| Inactive Lever | 1 st : $F(\text{vacc})_{1,148} = 4.423$ ($p < 0.05$) | -/- |
| | 2 nd : $F(\text{vacc})_{1,134} = 7.462$ ($p < 0.01$) | 1 ($p < 0.05$) |
| | 3 rd : $F(\text{vacc})_{1,148} = 6.969$ ($p < 0.01$) | -/- |
| | 4 th : $F(\text{vacc})_{1,113} = 24.523$ ($p < 0.0001$) | 8 ($p < 0.05$) |
| | 5 th : $F(\text{vacc})_{1,117} = 9.910$ ($p < 0.01$) | -/- |

Rats were vaccinated on 5 occasions during the maintenance phase (see section 4.3.2.5). Responses over 10 sessions following each vaccination were analysed by one-way ANOVA with repeated measures.

Although all 18 rats that entered the vaccination phase completed the schedule of vaccinations, the results presented for this phase are those for only 15 individuals. The 3 rats excluded from the analysis were lost during the subsequent withdrawal and reinstatement phases due to blocked catheters; however their lever-pressing responses during the vaccination phase were extremely variable, especially in the latter stages of this phase, presumably due to a blockage gradually developing in the catheter. As it was practically impossible to determine exactly at which point the development of the blocked catheter started to impact on lever-pressing behaviour, their data for this phase were excluded as too unreliable.

Figure 4.9: Effects of anti-cotinine vaccination on maintenance and extinction of self-administration behaviour



Response rates on the active lever (A), infusions obtained (B), and response rates on the inactive lever (C), during maintenance and extinction

Once response rates were stable under FR-3, rats were immunised with TT-CotSH or TT-Cysteine according to the schedule described in Chapter 2 (see section 2.2.3.4).

Self-administration continued throughout the immunisation period, except on injection days. After the final immunisation, rats were again required to reach stable levels of responding for 3 days before progressing to the extinction phase. During extinction, responses on the active lever were recorded, but had no programmed consequences. As well as removing the nicotine infusion, cues predicting the onset of nicotine infusion were also eliminated (infusion pumps turned off, stimulus light left on for the entire session).

Data shown are mean \pm S.E.M ($n = 7-8$ per group). The number of sessions between vaccinations varied among rats, resulting in large error bars for later time points; for the sake of clarity 10 sessions immediately after each vaccination (black arrows) and 13 sessions following initiation of extinction (grey arrow) are shown.

4.3.3.4 Extinction of self-administration behaviour

Once rats had completed the series of vaccinations and again fulfilled stable maintenance criteria (see section 4.3.2.4), lever-pressing responses on the active lever were extinguished by removing the nicotine-reinforcement (grey arrow in Figures 4.9 A – C indicates initiation of extinction), as well as the cues associated with nicotine administration (see section 4.3.2.6). As mentioned above, 3 rats were lost during this phase and the subsequent one, leaving 15 rats that completed the entire experiment.

Extinction was observed to be similar in both groups of rats, with active lever response rates decreasing to a level similar to the response rate on the inactive lever (see Figures 4.9 A – C).

4.3.3.5 Reinstatement of nicotine-seeking behaviour by nicotine and associated cues

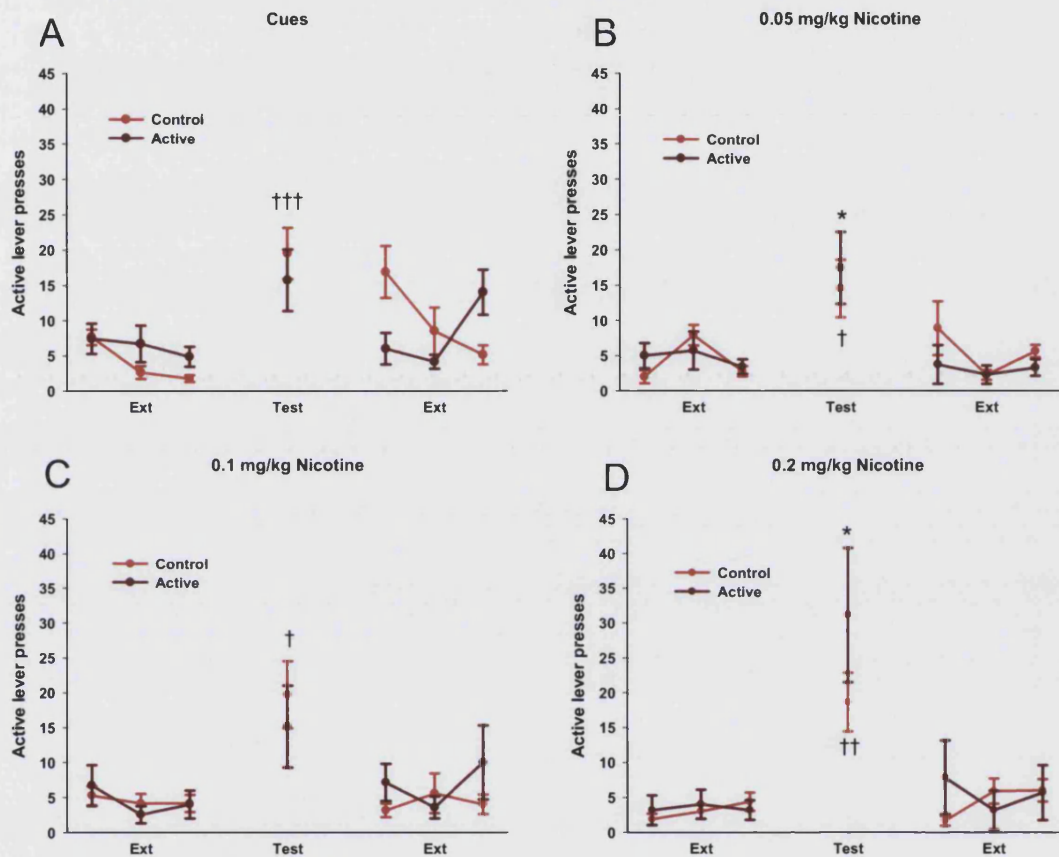
Reinstatement tests were carried out in the remaining 15 rats: nicotine-induced reinstatement was examined by injecting nicotine (0.05 – 0.2 mg/kg, s.c.) just prior to the self-administration session (no cues or nicotine infusions), whilst cue-induced reinstatement was explored by injecting saline and presenting only the cues (auditory & visual) associated with nicotine infusion (again no nicotine infusions).

Due to the mislabelling of vials containing a fresh batch of conjugates for vaccination (see section 2.3.2.3.4), several rats were mis-vaccinated. As a consequence, several "control" rats started to develop anti-cotinine antibody titres, whilst the titres in some rats in the "active" vaccine group did not increase as anticipated (see Figure 2.8 & section 2.3.3.3). Rats were assigned to an overall active or control group, depending on whether they had raised sustained anti-cotinine antibody titres or not (see section 2.3.3.3). It is these groups that the comparisons below refer to.

In rats vaccinated with control vaccine (TT-Cysteine), response levels during the reinstatement sessions (see Figures 4.10 A – D) were significantly greater compared to the baseline response levels of the same group at all nicotine doses tested ($p < 0.05$ for 0.05 – 0.1 mg/kg and $p < 0.01$ for 0.2 mg/kg), as well as with cues alone ($p < 0.001$). In the group vaccinated against cotinine (TT-CotSH), a significant reinstatement of lever-pressing behaviour (compared to the

baseline of this group) was only observed at the 0.05 and 0.2 mg/kg nicotine doses ($p < 0.05$).

Figure 4.10: Effects of anti-cotinine vaccination on reinstatement of nicotine self-administration behaviour



A) Cue-induced reinstatement; B-D) Nicotine-induced reinstatement

Once lever-pressing behaviour had been extinguished, rats were challenged with (A) saline (s.c.) plus cues associated with nicotine infusion (pump noise on, house-light off) or (B,C,D) nicotine (0.05, 0.1, or 0.2 mg/kg) with no cues, in a randomised order.

During these sessions active lever presses were again recorded but had no programmed consequences. After each challenge, rats were again required to reach extinction criteria prior to the next reinstatement test.

Data shown are mean \pm S.E.M, $n = 6-8$ per group; Test day response significantly different from mean baseline (3 days before): Control vaccine: $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$, $^{\dagger\dagger\dagger}p < 0.001$; Active vaccine: $^*p < 0.05$

When comparing the levels of active lever responding during reinstatement tests, no significant differences were observed between rats vaccinated against

cotinine and those vaccinated with control vaccine. Cue-induced response levels (see Figure 4.10 A) were similar to those elicited by the lowest nicotine dose (see Figure 4.10 B). At the highest dose tested (see Figure 4.10 D), the active vaccine group showed slightly higher levels of responding on the active lever than the animals vaccinated with TT-Cysteine, however this was not significant.

4.3.4 Discussion

Overall, the length of time required for rats to complete all phases of the self-administration experiments was much greater than anticipated, which was mainly due to the extremely slow acquisition of nicotine self-administration behaviour. This naturally had an impact on catheter patency, and the majority of rats were lost due to a failure to acquire lever pressing responses or the development of a block in the intravenous catheter. Nevertheless, upon completion of the experiments, several rats still had patent catheters – some 8-9 months after surgery – which was really quite remarkable.

Acquisition of nicotine self-administration

Intra-venous infusion of nicotine has been found to serve as a reinforcer of self-administration behaviour in rats, dogs and monkeys (Goldberg et al 1983). Acquisition of nicotine self-administration can be influenced by the dose of nicotine infused, with self-administration observed with doses of 0.03 - 0.06 mg/kg/infusion, but not 0.01 mg/kg/infusion, but the feeding schedule used also appears to play a role (Donny et al 1998). Food shaping (i.e. initially training rats to lever-press for food before switching to nicotine) has been shown to influence the speed of acquisition of nicotine self-administration, and appears to result in higher levels of responding for nicotine in rats maintained at relatively low body weights (Corrigall & Coen 1989, Donny et al 1995 & 1998) compared to free-feeding subjects (Shoaib et al 1997). However, neither weight restriction nor food deprivation is necessary to establish self-administration behaviour (Donny et al 1998). We did not use food shaping to establish lever-pressing behaviour, as we wanted to study the ability of nicotine to elicit self-administration (ie nicotine-seeking) behaviour without the prior association of the lever with a food reward.

The slow acquisition of nicotine self-administration observed in our study contrasts with previous reports of rats acquiring lever pressing behaviour for nicotine infusions in the space of approximately 14 - 40 sessions (Shoaib et al 1997 & 2003, Donny et al 1998, Caggiula et al 2001, Liu et al 2006). Studies by various groups have demonstrated reliable acquisition of self-administration with a nicotine dose of 0.03 mg/kg/infusion (Shoaib et al 1997, Donny et al 1998, Watkins et al 1999, Liu et al 2006); however, our animals did not appear to self-administer nicotine as readily. Cox et al (1984) observed that a reduction of the nicotine dose delivered with each infusion (0.03 to 0.003 mg/kg/infusion) resulted in a differential increase in active lever responses relative to responding on the inactive lever. We therefore attempted to increase the level of responses on the active lever and speed up the acquisition rate by switching the nicotine infusion dose from 0.03 to 0.015 mg/kg/infusion. After 2 days, having observed no effect on response rates, we reverted back to the established 0.03 mg/kg/infusion dose.

Sustained self-administration may result from a combination of heightened positive reinforcement early in the session, due to resensitisation of nAChRs over the 23-h abstinence period, and the need to reduce withdrawal by desensitising the overactive cholinergic system (Caggiula et al 2001). Consistent with this idea, the first cigarette of the day has been reported to possess enhanced reinforcing effects (Fagerström & Schneider 1989). We reduced the frequency of self-administration sessions from daily to every other day, effectively increasing the length of the abstinence period, and observed greater and more stable levels of responding as a result. This could have been due to rats being effectively "withdrawn" in between sessions as detailed above, however this seems unlikely as self-administration sessions only lasted 1 h and infusion rates were often not very high. Also, Watkins et al (1999) have reported that rats self-administering nicotine in 1 h daily sessions do not appear to be dependent on nicotine 24 h later, as indicated by the absence of somatic behavioural signs of withdrawal after a subcutaneous injection of mecamylamine. As less frequent self-administration sessions also meant that data acquisition was very much slower, we reverted back to daily sessions after a couple of weeks, since our planned experiments were comparatively lengthy

and we did not know how long the intravenous catheters were likely to remain patent.

Other possible factors that might have played a role in the lack of acquisition of nicotine self-administration behaviour include the rat strain used, and the time of day at which the experiments took place. Due to other ongoing studies unrelated to this thesis, the equipment used for the self-administration experiments could not be accessed until ~2pm (lights on 0700 – 1900 h). The time of day at which various research groups performed their self-administration studies does not appear to be well documented, however, one group report using 5 h sessions commencing at 9 am (Lynch & Carroll 1999). A chronobiological effect might therefore be at least partly responsible for the problems we experienced in terms of lack of acquisition of lever-pressing behaviour and variability of response rates.

The ability to acquire nicotine self-administration behaviour also appears to be partly dependent on genetic background. Shoaib et al (1997) explored this issue in an experiment comparing 4 different strains of laboratory rat: two outbred strains (Sprague-Dawley (SD) & Long-Evans (LE)) and two inbred strains (Lewis & Fisher (F344)). They observed that the outbred strains acquired the self-administration behaviour, whereas the two inbred strains failed to do so. These findings are supported by other studies, showing that SD (Donny et al 1995, Tessari et al 1995) and LE rats (Corrigall & Coen 1989) self-administer nicotine, while Fisher rats do not (Dworkin et al 1993). However, Wistar (Lynch & Carroll 1999, Watkins et al 1999), Holtzman (LeSage et al 2002) and hooded Lister rats (Shoaib et al 2003), which we were using, have also been shown to reliably self-administer nicotine.

Maintenance and extinction of nicotine self-administration behaviour

Response rates during the stable maintenance and vaccination phase of the experiment remained rather variable from one day to the next, but viewed over a longer period of time they were comparatively stable. Shoaib et al (2003) also observed relatively stable levels of responding over a ~40 session maintenance period.

We had originally intended to establish a dose-response curve for nicotine once rats had completed the vaccination schedule, by varying the concentration of

the nicotine solution infused, but had to abandon this plan due to the length of time taken for rats to progress through the acquisition phase, as well as the day to day variability of response rates. Several laboratories have previously reported obtaining a bell-shaped dose-response curve when dose per infusion was varied (0.002 - 0.12 mg/kg/infusion), with a maximum response around 0.01 - 0.03 mg/kg/infusion (Corrigall & Coen 1989, Shoaib et al 1997, Watkins et al 1999). This dose-response curve was observed to be relatively flat, as animals appeared to only partially compensate for the change in dose (Corrigall & Coen 1989, Donny et al 1995, Tessari et al 1995, Watkins et al 1999). In contrast, when cocaine doses were manipulated, animals compensated for the change in dose to obtain a relatively constant level of intake (Lynch & Carroll 1999).

When saline was substituted for nicotine and nicotine-associated cues were removed, responding on the active lever markedly reduced over approximately 6 - 17 sessions, which is similar to the 12-session period reported previously (Caggiula et al 2001, Liu et al 2006). Withdrawal of nicotine, or its pharmacological blockade, typically results in significant decreases in self-administration but not complete cessation of operant responding (Rose & Corrigall 1997, Shoaib & Stolerman 1999, Di Chiara 2000, Caggiula et al 2001). This may reflect a shift in the control of self-administration behaviour from the motivational effects of nicotine to a more automatic response, whereby stimuli conditioned to the drug trigger self-administration behaviour, which is relatively independent of the drug (Di Chiara 2000).

Reinstatement of nicotine self-administration

Smoking-related environmental stimuli have been implicated as an important factor in triggering relapse in abstinent tobacco smokers (Balfour et al 2000, Caggiula et al 2001, Liu et al 2006). In a study on briefly abstinent smokers, Rose and colleagues (Rose et al 2000) observed that cues alone (smoking of a de-nicotinised cigarette) increased smoking satisfaction to the same degree as smoking a nicotine-containing cigarette, but intravenous nicotine administration did not influence satisfaction. However, the reduction in craving due to smoking the de-nicotinised cigarette was greater in combination with intravenous nicotine

administration, so that both together produced a reduction in craving equal to that of smoking a regular nicotine-containing cigarette.

In animal self-administration experiments, the infusion of nicotine is typically paired with contextual stimuli such as lights (Corrigall & Coen 1989, Watkins et al 1999, Caggiula et al 2001, Liu et al 2006), and presentation of these contextual stimuli appears to promote nicotine taking and nicotine seeking responses. Rats have been observed to spontaneously recover previously extinguished behaviour upon re-exposure to the nicotine self-administration context after being maintained in their home cages for 21 days (Shaham et al 1997). Reintroduction of nicotine-paired stimuli after extinction also results in increased responses on the active lever (Caggiula et al 2001, LeSage et al 2004, Liu et al 2006).

In agreement with these findings, we observed a significant reinstatement effect of nicotine-associated cues in the group vaccinated with TT-Cysteine.

Significant reinstatement was also seen in this group following administration of various priming doses of nicotine, with a slightly larger effect at the highest dose. The group vaccinated against cotinine also demonstrated significant reinstatement in response to nicotine, but only at the highest and lowest doses used. Nicotine-induced reinstatement of nicotine self-administration behaviour does not appear to be as robust as that induced by nicotine-associated cues alone. While some studies have reported the reinstatement of previously extinguished nicotine self-administration behaviour by priming injections of nicotine in rats (Chiamulera et al 1996, Shaham et al 1997), LeSage and colleagues (LeSage et al 2004) observed that nicotine alone generally failed to reinstate nicotine self-administration, but that a significant reinstatement was achieved in combination with nicotine-associated cues. Similar results were obtained by Caggiula et al (2001), although this group utilised intravenous nicotine infusion, rather than priming injections, to test for nicotine-induced reinstatement (i.e. reacquisition).

Effects of vaccination on nicotine self-administration in rats

Vaccination against nicotine has been shown to affect the acquisition of nicotine (but not cocaine) self-administration behaviour under a 23-h access schedule, with vaccinated rats earning 38 % fewer infusions during the last week of a 3-

week training period (LeSage et al 2005). The percentage of rats meeting acquisition criteria was also lower in the vaccinated group than in the controls (36 % versus 70 %), but this was not statistically significant. Vaccination of rats during the maintenance phase did not affect nicotine self-administration until after the fourth dose, when the number of infusions obtained was significantly reduced (by ~57 %) in vaccinated rats compared to controls (LeSage et al 2005). The delayed onset of the effect was thought to be due to the slow development of antibody levels. Food-maintained responding was unaffected by anti-nicotine vaccination. Finally, while control rats exhibited a significant increase in responding in reinstatement tests following a low priming dose of nicotine, rats vaccinated against nicotine (during the extinction phase) did not, indicating that vaccination blocked the ability of a low dose of nicotine to reinstate nicotine self-administration behaviour (Lindblom et al 2002).

Towards the beginning of this trial, a new batch of cotinine derivative (CotSH) was synthesised, and fresh conjugates were produced (see sections 2.3.2.1.1 & 2.3.2.1.2). Unfortunately, vials containing the new conjugates for vaccination were mislabelled prior to their arrival in Newcastle. As a result of this error, several control rats were vaccinated with active vaccine and vice-versa, which resulted in relatively low anti-cotinine titres (~1:400) in the active group (more than 10-fold lower than those achieved with anti-nicotine vaccination in the studies mentioned above), as well as a small degree of anti-cotinine antibody production in the control animals (see Figure 2.9 & section 2.3.3.3).

Anti-cotinine vaccination did appear to increase lever-pressing rates, and some significant differences were observed between the vaccinated and control groups during the maintenance phase of the experiments, despite the overall variability in responding over time. Correlations between the number of responses on the active lever during reinstatement tests and the final titres of rats in the active group were examined to see if there was a trend towards increased lever pressing with higher titres, but interactions were found to be very weak ($r^2 = 0.0449$ (cues), $r^2 = 0.0258$ (0.05 mg/kg nicotine), $r^2 = 0.2259$ (0.1 mg/kg nicotine), and $r^2 = 0.1683$ (0.2 mg/kg nicotine)). This was at least partly due to the low number of subjects ($n = 6$) in the active group, which reached this late phase of the experiment.

4.4 Effects of anti-cotinine vaccination on conditioned taste aversion

4.4.1 Introduction

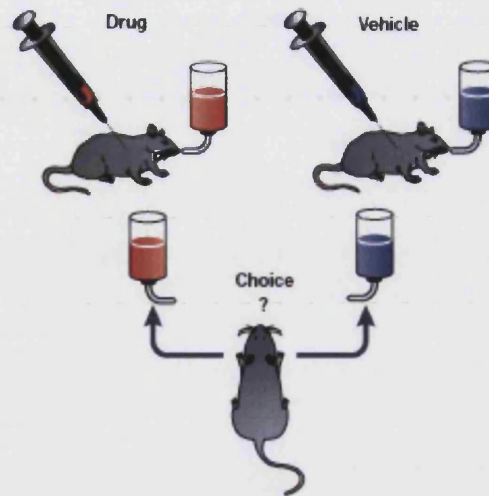
Nicotine is known to induce feelings of pleasure and reward in humans and other species. But like many other addictive drugs, it also has potent, aversive, unpleasant effects. Many people experience noxious effects such as nausea, coughs and dizziness on their initial experience with tobacco, which are attributed to nicotine, however, tolerance to the aversive effects of nicotine develops with repeated exposure. Although the precise neurobiological mechanism(s) underlying this tolerance to nicotine aversion are not known, its occurrence suggests that chronic nicotine exposure might induce functional alteration(s) in the neural systems mediating the aversive and/or rewarding effects of nicotine (Laviolette & van der Kooy 2004).

In rats, the aversive effects of nicotine appear to be dependent on direct stimulation of DA neurons in the mesolimbic pathway (see Chapter 1), whereas indirect stimulation of DA release via GABAergic / glutamatergic mechanisms may be implicated in mediating the motivational effects of nicotine, such as nicotine self-administration (Laviolette & van der Kooy 2004). This may be explained, at least in part, by differences between behavioural models: in nicotine self-administration drug delivery is contingent upon a behavioural response (lever-pressing), whilst in conditioned place preference/aversion tasks nicotine is administered non-contingently (Wonnacott et al 2005).

The aversive effects of nicotine have been implicated in the regulation of drug intake, perhaps by setting an upper limit to the amount consumed (Shoaib et al 2003). Aversive stimulus effects of nicotine are often examined using the conditioned taste aversion (CTA) procedure (Laviolette & van der Kooy 2004). CTA is believed to tap directly into the aversive properties of a drug and takes advantage of the fact that animals appear able to associate specific tastes with aversive states. By pairing a given drug with a particular taste, animals learn to avoid this taste, as the unpleasant effects of the drug become associated with it (Kumar et al 1983, Iwamoto & Williamson 1984). On the other hand, a taste that is paired with the injection of vehicle is not avoided when the animal is given a choice between the two flavours (see Figure 4.11).

Figure 4.11: Conditioned taste aversion procedure

On conditioning days, rats are given access to one of two flavoured solutions, followed by administration of drug or vehicle. Following several training sessions with each injection-flavour pairing, rats are given access to both flavoured solutions at the same time in a test session (2-bottle choice test). The amount of each solution consumed is measured, with a mean score significantly lower than 50 % indicating a conditioned taste aversion to the drug-paired solution. (from Laviolette & van der Kooy 2004)



Pavlovian conditioning appears to play a role in the development of the association between a flavoured solution (conditioned stimulus) and drug effects (unconditioned stimulus) (Bevins & Palmatier 2003). In taste avoidance and place conditioning preparations, drug pre-exposure is thought to alter an affective process (e.g. aversive or appetitive effect of nicotine), which in turn becomes associated with the conditioned stimulus (flavour). Several studies have used nicotine as a drug stimulus in this task and have reported that, like many other addictive drugs, it produces potent aversive effects (Shoaib et al 2000 & 2002, Laviolette & van der Kooy 2003, Pescatore et al 2005). Such reports, as well as studies using self-administration or conditioned place preference/aversion (CPP/A) procedures, have provided further evidence of the dual nature of the motivational effects of nicotine in animals, consistent with the reported aversive and rewarding psychological effects of nicotine in humans (Laviolette & van der Kooy 2004).

Nicotine conditioned taste aversion (CTA) has been observed in rats utilising a one-bottle test CTA procedure, where the drug is paired with only one flavoured solution, and saline is paired with water (Pescatore et al 2005). However, with this set-up there is a risk that any aversion observed might be due to a dislike of the flavour the drug was paired with, rather than the effects of the drug itself. With the two-bottle procedure (Shoaib et al 2000 & 2002), a selective avoidance of the drug-paired flavour indicates that the aversion is a function of the

association of the taste with the effects of nicotine, rather than a generalised unconditioned suppression due to preference of one flavour over another (Pescatore et al 2005).

Smokers appear to subconsciously titrate their nicotine intake to achieve a desired blood concentration, taking deeper and longer inhalations if they are switched to a cigarette brand with a low nicotine yield, and limiting their nicotine intake through more shallow inhalations when smoking a higher-yield brand (Russell et al 1980, West et al 1984). The existence of this upper limit of nicotine intake in order to achieve a given blood nicotine concentration could be an indication of possible aversive effects of nicotine above a certain level.

Removing or reducing cotinine-mediated antagonism of nicotine's actions by means of vaccination against cotinine could lead to a reduction in the number of cigarettes smoked per day in current smokers, which might assist in a reduce-to-quit approach to smoking cessation. However, it could also help to enhance the efficacy of NRT at relieving nicotine withdrawal symptoms in those trying to quit the habit. Here we examine the effects of anti-cotinine vaccination on the development of aversion to a nicotine-paired solution in rats, using a two-bottle conditioned taste aversion procedure.

4.4.2 Methods

4.4.2.1 Animals & apparatus

Male hooded Lister rats (Harlan, UK), 6 - 7 weeks old, were housed in groups of 4 for the duration of the vaccination process with access to food and water *ad libitum*. Prior to the conditioned taste aversion experiments, rats were housed individually and acclimatised to these conditions for one week.

In preparation for the conditioned taste aversion experiments on vaccinated animals, two small pre-trials were undertaken to establish the nicotine test doses to be used in the experiments on vaccinated rats. For the unvaccinated pre-trials, rats (6 - 7 weeks old) were acclimatised to the animal unit for a week, before being housed individually for another week prior to the start of the experiments.

Rats were weighed on a daily basis throughout the time they were housed individually, in order to monitor their weight and condition and determine injection volumes.

All conditioned taste aversion experiments were carried out in the rats' home cages, using 50 ml graduated conical tubes (instead of the usual drinking bottles) and rubber stops with drinking spouts to facilitate accurate measurement of fluid intake. Three sets of tubes were used: one for each flavoured solution, and another set for water.

4.4.2.2 Conditioned taste aversion (CTA) procedures

Once rats had been housed individually for a week, their access to water was restricted to 1 h per day (1 – 2 p.m.). Rats were adapted to this regime for 7 days, before any presentation of the flavoured solutions.

Then, during the hour in which the rats had previously had access to water, one of two flavoured solutions (sodium saccharin 0.1 % or sodium chloride 0.9 %) was presented for 15 min on every second day. (On the days in between, rats were given access to water for 1 h as during the training period.) The two flavoured solutions were presented alternately, thus each flavour was presented to a given rat every fourth day. Immediately after the tubes containing the flavoured solutions were removed, the rats were injected with either nicotine (0.05, 0.1, 0.2 or 0.4 mg/kg, s.c.) or saline (flavour-injection pairing) and returned to the home cage. For half of the rats, one flavour was consistently paired with nicotine, whereas the other flavour was paired with vehicle. These arrangements were reversed in the remaining rats to balance out effects due to inherent palatability of the flavours. The order of nicotine and saline injections was also counterbalanced.

An additional 15 ml of water (10 ml for the smaller animals used in the pre-trials) was presented 3 - 4 h after removing the flavoured solutions on conditioning days, in order to prevent dehydration and weight loss. Each rat was given two drug and two saline conditioning sessions (4 of each for the CTA experiments on vaccinated rats).

2 days after completing the conditioning sessions, rats were subjected to a two-stimulus test, consisting of simultaneous presentation of both nicotine-and saline-paired flavoured solutions for 15 min. The next day, the test was repeated with the positions of the two stimuli (flavours) reversed, in order to balance for side preferences.

4.4.2.3 Data analysis

The percentage of nicotine-paired flavoured solution consumed during the two-bottle tests was subjected to arc-sine transformation to normalise the distribution. To test for a significant CTA for each group, t-tests were carried out to determine whether the means differed significantly from 50 %. A mean score near to 50 % would indicate that the drug did not produce conditioned taste aversion or preference.

4.4.3 Results

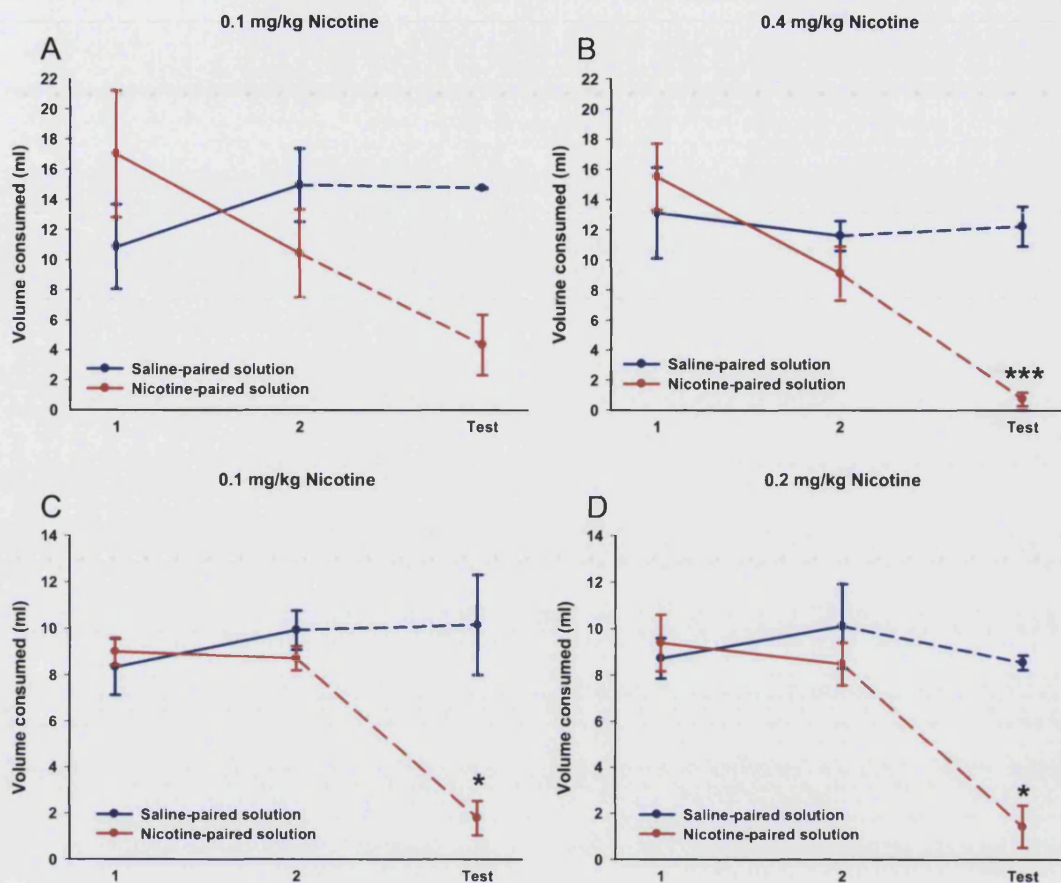
4.4.3.1 Optimisation of nicotine dose (pre-trials)

Two pre-trials were conducted to establish the nicotine doses to be used in the experiments on the vaccinated animals. Nicotine was tested at doses of 0.1, 0.2 and 0.4 mg/kg (s.c.), with each group of rats being assigned one dose.

During conditioning sessions, rats were alternately given access to one of two flavoured solutions: one paired with an injection of nicotine, and the other paired with saline, as described in the methods. After 2 conditioning sessions for each solution, rats were given access to both flavoured solutions simultaneously (without injections) to test for conditioned taste aversion.

Rats were trained to drink water during a daily 1 h access session, and consumed on average around 16 ml/day in the first pre-trial, and around 13 ml/day in the second pre-trial (data not shown). Once the conditioning sessions (flavoured solutions paired with injections) began, this decreased to 13 ml/day and 9 ml/day, respectively.

During the first set of conditioning sessions (i.e. first exposure to the flavoured solutions), rats showed no preference/aversion: consumption of nicotine- and saline-paired solutions was very similar, with rats in the first pre-trial (see Figures 4.12 A & B) drinking ~14 ml, and those in the second pre-trial (see Figures 4.12 C & D) drinking ~9 ml. In the first pre-trial, the 0.1 mg/kg dose of nicotine did not produce a CTA ($p = 0.0673$), despite a trend in that direction, whereas at the 0.4 mg/kg dose a significant CTA was observed ($p = 0.0007$; see Figures 4.12 A & B). We therefore conducted the second pre-trial with a lower dose of nicotine, which produced similar results (see Figures 4.12 C & D), however, this time rats exhibited significant CTA at both nicotine doses (0.1 and 0.2 mg/kg) tested ($p = 0.0484$ and $p = 0.0153$, respectively).

Figure 4.12: Optimisation of nicotine dose in conditioned taste aversion pre-trials

A & B) Pre-trial 1: CTA produced by 0.1 and 0.4 mg/kg nicotine; C & D) Pre-trial 2: CTA produced by 0.1 and 0.2 mg/kg nicotine

Two flavoured solutions were presented alternately, with each rat receiving its assigned dose of nicotine (0.1, 0.2 or 0.4 mg/kg, s.c.), or saline, immediately after removing the flavoured solutions, and then being returned to their home cage. Flavour-drug pairings were counterbalanced to balance out effects of palatability of the two flavours. The order of nicotine and saline injections was also counterbalanced. An additional 10 ml of water was presented 3-4 h after removing the flavoured solutions on conditioning days, to prevent dehydration and weight loss. Each rat was given two drug and two saline conditioning sessions prior to the two-bottle choice tests (see section 4.4.2.2).

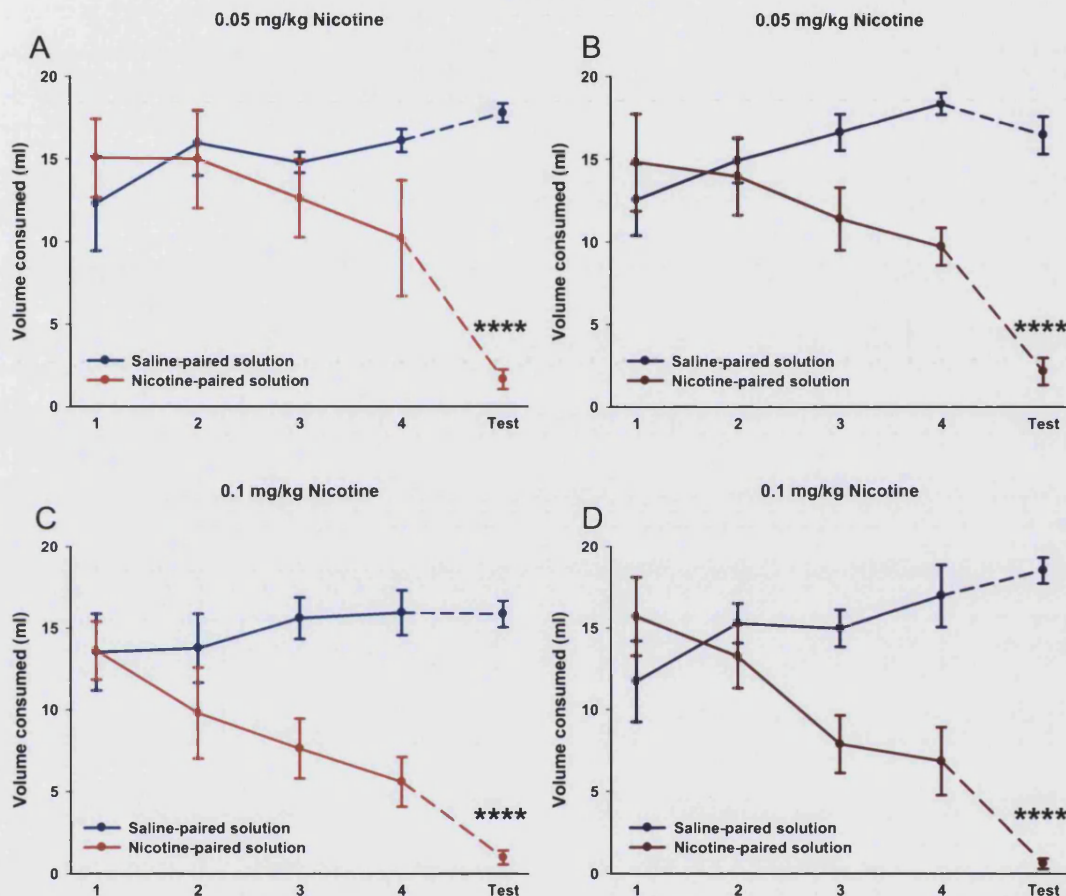
Data shown are mean \pm S.E.M, $n = 4$ per group; * $p < 0.05$, *** $p < 0.001$ (Significant difference in fluid intake from 50 %)

4.4.3.2 Effect of vaccination on CTA

Conditioning sessions were conducted as described above (see section 4.4.3.1); rats were exposed to each solution/injection pairing on four occasions

to maximise conditioning effects with the low nicotine doses used. Based on the results of the pre-trials, we examined doses of 0.1 and 0.05 mg/kg nicotine.

Figure 4.13: Effects of anti-cotinine vaccination on conditioned taste aversion to nicotine



CTA produced by 0.05 mg/kg nicotine in control (A; TT-Cysteine) and immunised (B; TT-CotSH) groups; CTA produced by 0.1 mg/kg nicotine in control (C; TT-Cysteine) and immunised (D; TT-CotSH) groups; Data shown are mean \pm S.E.M, $n = 6-8$ per group; **** $p < 0.0001$ (Significant difference in fluid intake from 50 %)

Two flavoured solutions were presented alternately, with each rat receiving its assigned dose of nicotine (0.05 or 0.1 mg/kg, s.c.), or saline, immediately after removing the flavoured solutions, and then being returned to their home cage. Flavour-drug pairings were counterbalanced, as was the order of nicotine and saline injections. An additional 15 ml of water was presented 3-4 h after removing the flavoured solutions on conditioning days, to prevent dehydration and weight loss. Each rat was given 4 drug and 4 saline conditioning sessions prior to the two-bottle choice tests.

Water consumption during the initial training averaged around 16 ml/day, which decreased to approximately 9 ml/day once conditioning sessions were initiated (data not shown). In the first conditioning session, the consumption of both nicotine- and saline-paired solutions was very similar at around 14 ml. Over the course of the 4 conditioning sessions, consumption of the nicotine-paired solution gradually decreased from one session to the next (see Figure 4.13). In the two-bottle choice tests, all groups demonstrated a highly significant conditioned taste aversion ($p < 0.0001$), despite the low concentrations of nicotine used in these experiments.

Vaccination against cotinine did not appear to affect the magnitude of the conditioned taste aversion to nicotine observed at either of the doses tested.

4.4.4 Discussion

Over the course of a week, rats were trained to drink their daily fluid intake within a 1-hour period. The development of conditioned taste aversion to flavoured solutions paired with injections of saline / nicotine over a range of doses was then examined in two pre-trials, before the effect of anti-cotinine vaccination on nicotine conditioned taste aversion were studied in a larger trial.

Fluid consumption

The variation in water consumption between trials is likely to be due to differences in the age and size of the animals. All rats on the drink deprivation schedule were closely monitored for weight loss and signs of dehydration, neither of which were observed. Animals tended to lose a couple of grams of body weight as a result of the initial change from 24-h to 1-h access to water, however they rapidly adjusted to the new access schedule and usually regained the weight initially lost by the end of the experiment.

Although weight differences within each trial were marginal, the rats used in the second pre-trial were several weeks younger than those used in the first pre-trial, and consequently slightly smaller (250 – 300 g versus 300 – 350 g), which could explain why their required daily intake of water was a little lower.

However, there appears to be an upper limit to daily fluid requirement, since the vaccinated rats, which were approximately 3 months older and also heavier

(450 – 550 g) than those used in the first pre-trial, did not drink noticeably more water during their training period.

Water consumption decreased by several millilitres once conditioning sessions started. Several hours after each conditioning session, rats were given access to a limited ration of water overnight (10 ml in the pre-trials, 15 ml for the vaccinated animals). The consumption of this overnight ration the night before the next 1-h water access session was likely to be responsible for the slight reduction in the amount of water drunk on the days in between conditioning sessions.

Conditioned taste aversion to nicotine

Pairing of nicotine administration (0.2 – 0.4 mg/kg, s.c.) with the consumption of a flavoured solution has been shown to produce a significant conditioned taste aversion in rats (Shoaib et al 2000), using the two-bottle choice test procedure. Similar results have also been obtained using mice (Gommans et al 2000). Our results are in agreement with these findings (see Figure 4.12) and show that the conditioned taste aversion resulting from the association of the taste of a flavoured solution with an injection of nicotine can be seen even with very low doses of nicotine (0.05 – 0.1 mg/kg; see Figures 4.12 & 4.13). The effect was especially strong when the number of conditioning sessions prior to the two-bottle choice tests was increased (see Figure 4.13).

Pre-treatment with DH β E (0.5 - 5.0 mg/kg, s.c.) 30 min prior to nicotine administration fails to block nicotine (0.4 mg/kg, s.c.) conditioned taste aversion (Shoaib et al 2000). Co-administration of DH β E (5.0 mg/kg, s.c.) together with nicotine (0.2 - 0.4 mg/kg, s.c.) on the other hand, prevented the development of conditioned taste aversion. This observed short-lasting competitive antagonism by DH β E suggests that aversive effects of nicotine may be mediated, at least in part, by α 4 β 2* nAChRs (Shoaib et al 2000).

Mice have also been shown to display a strong conditioned taste aversion to nicotine, which has been shown to be DH β E-sensitive in this species too (Gommans et al 2000). Further evidence for the involvement of α 4 β 2 nAChRs in mediating conditioned taste aversion comes from studies using knockout mice. Mice lacking the β 2 nAChR subunit show a strong reduction in nicotine-induced conditioned taste aversion compared to wild-type controls, indicating

that $\beta 2^*$ nAChRs are involved in mediating the aversive effects of nicotine (Shoaib et al 2002).

Conditioned taste aversion studies rely on systemic nicotine administration, which also targets peripheral nAChRs. The activation of such receptors has the potential to produce noxious and toxic effects, leading to the expression of conditioned taste aversion. The same problem occurs with intra-venous self-administration procedures, and high concentrations of intravenously administered nicotine have been observed to produce aversive effects as a result (Rose & Corrigall 1997). However, infusion of nicotine directly into the VTA (to exclude peripheral effects) also appears to produce both aversive and rewarding effects, depending on the concentration of nicotine administered (Laviolette & van der Kooy 2003). Examining the effects of nicotine infusion into various brain regions, Shoaib and Stolerman (1995) found that conditioned taste aversion was produced by bilateral nicotine administration directly into the nucleus accumbens, and that this effect was blocked in rats pre-treated with the nicotinic antagonist mecamylamine. Infusion of nicotine directly into the VTA, striatum, hippocampus and several other brain regions, on the other hand, failed to produce CTA. It therefore appears that the aversive effects produced by systemically administered nicotine may be mediated at least in part through nicotinic receptors located in the NAcc (Shoaib & Stolerman 1995).

Effects of anti-cotinine vaccination

By reducing cotinine-mediated antagonism of nicotinic effects, anti-cotinine vaccination should enhance the efficacy of NRT and increase the chances of a quit attempt being successful. Unfortunately, in this study anti-cotinine vaccination did not appear to result in an enhancement of the conditioned taste aversion to nicotine observed, compared with control-vaccinated rats (see Figure 4.13). However, it is very likely that the lack of a vaccination effect was due to the low anti-cotinine antibody titres achieved in this Trial (~1:800; see Figure 2.10, section 2.3.3.3) as a result of the mislabelling of vials containing the conjugates (see section 2.3.2.3.4), and the consequent mis-vaccination of rats.

As far as I am aware, none of the studies into anti-nicotine vaccines have examined effects on conditioned taste aversion, but titres achieved in other

behavioural studies showing effects of nicotine vaccines (Pentel et al 2000, Lindblom et al 2002, LeSage et al 2005) have been much higher ($> 1:10,000$) than those obtained here. The experiment therefore needs to be repeated in rats with higher titres of anti-cotinine antibodies in order to be able to assess the impact of such antibodies on the development of conditioned taste aversion to nicotine.

4.5 Effects of anti-cotinine vaccination on mecamylamine-precipitated withdrawal

4.5.1 Introduction

Chronic tobacco use generally results in a state of "physical dependence", where termination of drug administration results in an abstinence syndrome, comprising symptoms such as irritability, anxiety, depression, increased hunger, restlessness, difficulty concentrating, sleep disturbances, weight gain, decreased heart rate and craving for tobacco (Malin 2001). By relieving these symptoms, smoking acts as a "negative reinforcer", and has been implicated in the relapse of many smokers during a quit attempt. So, whilst positive reinforcement is the main factor in the acquisition and routine maintenance of the smoking habit, the abstinence syndrome and the ability of renewed smoking to provide negative reinforcement can play an important role during a cessation attempt (Malin 2001).

Development of a preclinical model to assess nicotine withdrawal

Malin and colleagues have developed a preclinical model of nicotine abstinence syndrome in the rat (Malin et al 1992), in which dependence is induced through continuous subcutaneous infusion of nicotine (1.05 or 3.15 mg/kg/day free base) via an osmotic minipump for 7 days, and abstinence can be initiated by terminating the infusion through pump removal. The result is an abstinence syndrome involving a pattern of behaviours resembling opiate abstinence in the rat (Malin et al 1992).

In order to quantify the severity of nicotine withdrawal, the number of occurrences of each sign is scored against a standard checklist of abstinence signs included teeth chattering, chewing, gasping, writhing, head shakes, body

shakes, tremors (particularly cheek tremors), ptosis and miscellaneous less frequent signs. The subject's overall abstinence score is defined as the number of signs cumulated across all categories (Malin et al 1992). The group termed these behavioural abstinence signs "somatic abstinence signs" to distinguish them from other behavioural changes, such as changes in trained or conditioned behaviour, but suggested that the term "somatic behavioural signs" might be more appropriate since some signs (such as vacuous chewing or a wet dog shake) were not so much changes in somatic state as an actual somatically expressed behaviour coordinated by the CNS (Malin 2001).

The procedure appears to be a relatively robust model of nicotine dependence, with evidence from several groups indicating that the observed abstinence behaviours are the result of a period of nicotinic receptor overstimulation followed by reduced nicotinic stimulation.

- Abstinence scores are greater following nicotine infusion than saline infusion, and increased with nicotine dose (Malin et al 1992, Hildebrand et al 1997). The amount of nicotine typically infused (1-3 mg/kg/day free base) did not appear to debilitate the rats during the infusion period in terms of locomotor activity, open-field behavioural observations, weight gain, food consumption or operant performance for food reward (Malin 2001).
- Significantly more abstinence signs are observed at 16 h after the end of drug infusion (by pump removal) than before infusion, during infusion or following a subsequent recovery period (Malin et al 1992).
- Nicotine abstinence can be potently and promptly reversed by injection of 0.4 mg/kg nicotine (Malin et al 1992).
- Administration of a nicotinic antagonist such as dihydro- β -erythroidine (Epping-Jordan et al 1998, Malin et al 1998), mecamylamine (Malin et al 1994, Hildebrand et al 1997), hexamethonium (Malin et al 1997) or chlorisondamine (Hildebrand et al 1997) also promptly precipitated the abstinence syndrome. Injection of mecamylamine or chlorisondamine even appeared to result in a larger increase in the abstinence score than termination of nicotine infusion by pump removal (Hildebrand et al 1997).

Nicotine replacement therapy (NRT) primarily improves cessation efforts by reducing the severity of withdrawal symptoms and cravings occurring as a

consequence of nicotine abstinence. It provides an alternative source of nicotine, avoiding exposure to various other carcinogenic components contained in cigarette smoke, and is the most commonly used medication for smoking cessation (Schneider et al 2001, Hughes 2003, Foulds et al 2004). The anti-cotinine vaccine is designed to make NRT more effective by removing or reducing any cotinine-mediated antagonism of nicotine's actions by initiating the production of cotinine-specific antibodies, which bind the metabolite and prevent it from crossing the blood-brain-barrier. We therefore used this animal model of nicotine withdrawal to examine the effects of anti-cotinine vaccination on the expression of a nicotine abstinence syndrome in nicotine-dependent rats.

4.5.2 Methods

4.5.2.1 Animals & apparatus

As a result of the mislabelling of vials containing fresh conjugate solutions for vaccination (see section 2.3.2.3.4), the "control" vaccinated rats had developed some (low) anti-CotSH antibody titres (see Figure 2.9 & section 2.3.3.3). We therefore decided to only use rats with the lowest (for "control" groups) and highest titres (for "vaccinated" groups) for the experiment.

Experiments were performed in the same set-up as the locomotor experiments (see Newcastle apparatus 4.2.2.1), with locomotor activity recorded as described. In addition to scoring somatic signs during the experiment (see 4.5.2.3), each box/rat was also filmed during the test using video cameras and a video recorder, and the tapes stored as a record and backup. The use of a split screen allowed all four boxes to be filmed simultaneously.

4.5.2.2 Minipump implantation

Rats were anaesthetised with isoflurane, and an incision made on one flank. After creating a skin pocket by means of blunt dissection, rats were implanted with a 2ML1 Alzet osmotic minipump filled with nicotine solution (to deliver 3.16 mg/kg/day nicotine base in physiological saline), which was administered as a 7-day continuous infusion, rendering the animal nicotine-dependent. Control animals were implanted with a "blank" (a piece of plastic of similar size, shape & weight as a minipump). The incision was closed using wound clips.

4.5.2.3 Behavioural observations

On day 7, each rat was injected subcutaneously with 1 ml/kg saline, placed in a locomotor activity box, and observed for 30 min. Rats were then challenged with mecamylamine HCl (Sigma) by subcutaneous injection (1 mg/kg), and observed for a further 60 min.

Rats were observed one at a time for 1 min periods (4 rats per run, so each was observed for 1 min every 4 min). Behaviours were scored against a checklist of behavioural signs associated with nicotine abstinence (developed and validated by Malin et al 1992). Categories included body and head shakes, cheek tremors, teeth chatters and chews, scratches, ptosis, eye blinks, foot and genital licks, and miscellaneous less frequent signs (yawns, escape attempts, gasps and writhes). Ptosis was scored no more frequently than once each minute (i.e. present/absent), and all other signs were scored as number of observed behaviour per minute, with any continuous behaviour (scratching, foot licking, tremors etc) counted no more than once every 15 sec. All sessions were captured on video (VHS) as backup.

In addition to the behavioural observations, the rats' locomotor activity was also monitored throughout the 90 min session (using the apparatus described in section 4.2.2.1).

At the end of these experiments rats were terminally anaesthetised and perfused with PBS (see section 3.2.2.2.2), and brain tissues used for radioligand binding and determination of blood and brain nicotine and cotinine levels (see Chapter 3).

4.5.2.5 Data analysis

Locomotor activity was recorded at 10 min intervals over the entire 90 min measurement period, and then summarised to give total activity in the baseline (1 - 30 min) and post-mecamylamine phase (31 - 90 min), respectively. A two-way ANOVA with factors vaccination (control vs active) and treatment (blank vs nicotine) was performed, followed by Bonferroni's post-hoc analysis.

Behavioural signs were scored as above (see section 4.5.2.3); they are presented as either total abstinence score (sum of all behaviours scored in a given period of time) or as scores for individual signs. Analysis of total scores (baseline, post-mec & post-mec minus baseline) and scores for individual signs

(post-mec minus baseline) was carried out using ANOVAs and Bonferroni's test as above.

4.5.3 Results

Locomotor activity

Locomotor activity measurements were divided into baseline activity (30 min prior to mecamylamine administration) and post-mecamylamine activity (60 min).

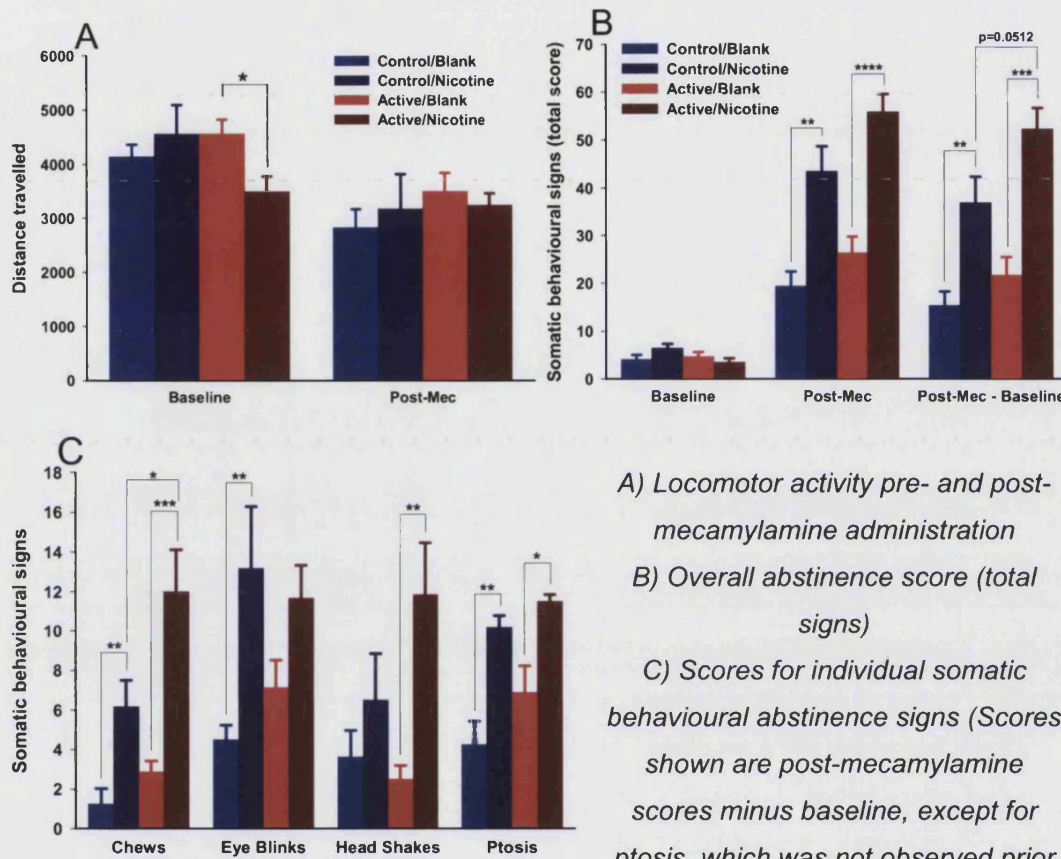
Baseline locomotor activity declined steadily over the course of the measurement period (data not shown). Activity levels (distance travelled) over the baseline period in the control groups (~4,000 (TT-Cysteine); ~4,500 (TT-CotSH); see Figure 4.14 A) were comparable to those of the control groups (~4,500 (TT-Cysteine), 4,800 (TT-CotSH)) in the first 30 min of the experiments on the effects of acute nicotine administration (see section 4.1 & Figure 4.5 B). Activity in the TT-Cysteine vaccinated group receiving a chronic nicotine infusion (~4,500) was similar to both of the control groups, whereas the nicotine-treated group immunised with TT-CotSH (~3,500) had much lower activity levels, which were more like the high-dose (0.4 mg/kg) nicotine groups in the acute nicotine administration study (~3,000 (TT-Cysteine); ~3,400 (TT-CotSH)). The difference in activity levels between the saline- and nicotine-treated TT-CotSH groups in this study was significant ($p = 0.0199$; see Figure 4.14 A), resulting in an overall significant interaction of vaccination and treatment ($F(\text{vaccination} \times \text{treatment})_{1,24} = 5.023$, $p = 0.0345$).

Activity levels continued to decline over time after mecamylamine administration (data not shown). No significant differences between any of the groups were observed (see Figure 4.14 A), although there was still a trend towards lower locomotor activity in the nicotine-treated, compared to the saline-treated TT-CotSH group; however, this was not significant ($p = 0.5643$).

Total abstinence score

Rats were observed during both the baseline and post-mecamylamine periods, and behaviours were scored against a checklist of behavioural signs associated with nicotine abstinence. The total abstinence scores (sum of all behaviours scored over each period) are shown in Figure 4.14 B.

Figure 4.14: Effects of anti-cotinine vaccination on the occurrence of somatic behavioural signs during mecamlamine-precipitated nicotine withdrawal



A) Locomotor activity pre- and post-mecamlamine administration

B) Overall abstinence score (total signs)

C) Scores for individual somatic behavioural abstinence signs (Scores shown are post-mecamlamine scores minus baseline, except for ptosis, which was not observed prior to mecamlamine administration in any of the groups)

Rats were implanted subcutaneously with either osmotic minipumps delivering 3.16 mg/kg/day nicotine (base) for 7 days or a "blank" (mock minipump). On day 8, rats were given an injection of saline and placed in locomotor activity boxes for 30 min, where behavioural signs were observed and scored.

They were then injected with 1 mg/kg mecamlamine (s.c.) and returned to the activity boxes and scored for behavioural signs for a further 60 min.

The number of occurrences of each sign was recorded, and the subject's overall abstinence score (total score) defined as the number of signs cumulated across all categories in a given period of time (e.g. post-mecamlamine administration).

Data presented are mean \pm S.E.M, $n = 6-8$ per group; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

Baseline scores: No differences were observed between groups in terms of baseline abstinence scores, i.e. prior to mecamylamine administration (see Figure 4.14 B).

Post-mecamylamine scores: Despite the low anti-CotSH antibody titres achieved in this trial (~800), due to the mislabelling of the fresh batch of conjugates and subsequent mis-vaccination of rats (see sections 2.3.2.3.4 & 2.3.3.3), total scores were higher in the nicotine-treated group vaccinated against cotinine than in the nicotine-treated TT-Cysteine group. Analysis of the post-mecamylamine total scores revealed a significant main effect of vaccination ($F(\text{vaccination})_{1,24} = 6.421$, $p = 0.0182$), and a highly significant main effect of treatment ($F(\text{treatment})_{1,24} = 49.732$, $p < 0.0001$). Post-hoc test showed significantly increased scores for nicotine pre-treated rats compared to the corresponding controls, with a greater difference between the TT-CotSH groups than the groups vaccinated with TT-Cysteine ($p < 0.01$ and $p < 0.0001$ for control and active vaccine groups, respectively). The difference between the two nicotine-treated groups, however, was not significant ($p = 0.0832$; see Figure 1.14 B).

Post-mecamylamine minus baseline: Both the treatment and vaccination effects were still seen when the baseline scores were subtracted from the post-mecamylamine scores ($F(\text{vaccination})_{1,24} = 7.190$, $p = 0.0131$ and $F(\text{treatment})_{1,24} = 41.585$, $p < 0.0001$). Post-hoc test showed significantly increased scores for nicotine pre-treated rats (see Figure 4.14 B) compared to those implanted with a blank ($p < 0.01$ and $p < 0.001$ for control and active vaccine groups, respectively); again the difference was greater between the two TT-CotSH vaccinated groups. The increase in somatic behavioural abstinence signs in nicotine-treated rats vaccinated against cotinine, compared to those receiving control vaccine, was also very nearly significant ($p = 0.0512$).

Individual somatic signs

The scores for the four signs that accounted for the majority of the total abstinence score (89.5 - 99.8%) are shown in Figure 4.14 C. The remaining abstinence signs occurred quite rarely in any of the groups.

Chews: In the "chews" category, significant main effects of vaccination and treatment were observed ($F(\text{vaccination})_{1,24} = 9.648$, $p = 0.0048$ and

$F(\text{treatment})_{1,24} = 34.198, p < 0.0001$). Post-hoc analysis revealed significant increases in the number of chews in nicotine-treated rats compared to their control group ($p < 0.01$ (TT-Cysteine); $p < 0.001$ (TT-CotSH)). The difference was again greater in the group vaccinated against cotinine, and a significant increase in chews was also observed in the nicotine-treated TT-CotSH, compared to the nicotine-treated TT-Cysteine group ($p < 0.05$).

Eye blinks: A treatment effect was again observed ($F(\text{treatment})_{1,24} = 13.991, p = 0.0010$). Although the number of eye blinks observed was increased in both nicotine-treated groups, compared to their controls, the difference was only significant in the TT-Cysteine vaccinated groups ($p < 0.01$).

Head shakes: For "head shakes" a main effect of treatment was also observed ($F(\text{treatment})_{1,24} = 12.259, p = 0.0018$), but this time the increase in scores was only significant in the TT-CotSH vaccinated groups ($p < 0.01$).

Ptosis: Ptosis was only observed after mecamylamine administration, but a significant main effect of treatment was observed for this abstinence sign, too ($F(\text{treatment})_{1,24} = 23.725, p < 0.0001$). The post-hoc analysis revealed significant increases in both nicotine-treated groups, compared to their control groups, with a greater increase in the TT-Cysteine group ($p < 0.01$), compared to the group vaccinated against cotinine ($p < 0.05$).

4.5.4 Discussion

Comparison between the rat model of nicotine abstinence syndrome and withdrawal in human smokers

There are obvious differences between this rat model and the clinical situation experienced by smokers. For reasons of practicality, the period of nicotine exposure is shortened to 1-2 weeks, compared with years of smoking. To compensate for this, the rate of nicotine exposure is quite high. At the 1 mg/kg/day rate, the amount of nicotine administered is comparable to the amount ingested by a 70 kg smoker smoking three packs of average-yield cigarettes per day, whereas at the 3 mg/kg/day rate, the amount administered is comparable to that ingested by a 5 pack/day smoker of high-yield cigarettes (Malin 2001).

Another difference is the continuous and subcutaneous drug administration in the laboratory model, compared to the intermittent administration by inhalation

in smoking. However, it is well established that many heavy smokers "titrate" their smoking consumption to maintain relatively stable levels of serum nicotine during waking hours (Malin 2001).

A further important point to bear in mind is that rats generally have much higher metabolic and drug clearance rates than humans. Blood nicotine concentration can therefore provide a more meaningful basis of comparison: a blood nicotine concentration of 40 ng/ml has been measured in rats infused with ~3 mg/kg/day nicotine for 7 days (Malin 2001), as well as in rats infused with 4 mg/kg/day for 14 days (Sanderson et al 1993). This is virtually identical to the concentration maintained by human subjects smoking 30 high-yield cigarettes per day during waking hours (Benowitz et al 1982).

Characteristics of the nicotine abstinence syndrome in rats

Several groups have observed decreased locomotor activity as a result of nicotine withdrawal in rats previously infused with nicotine (~3 mg/kg/day, free base) for 7 (Hildebrand et al 1997) or 14 days (Fung et al 1996) compared to saline-infused animals, when using pump removal (spontaneous withdrawal) to initiate nicotine abstinence. However, when withdrawal was induced by injection of a nicotinic antagonist (mecamylamine or chlorisondamine), Hildebrand and colleagues did not see a decrease in locomotor activity in nicotine-treated rats, compared to those pre-treated with saline (Hildebrand et al 1997). This is also in agreement with our results, as we observed no significant effect of mecamylamine on locomotor activity.

Administration of mecamylamine did, however, induce a withdrawal syndrome in all groups, which emerged gradually, with only few signs detected in the first 5 min after the injection, in agreement with Hildebrand et al (1997). A high dose of mecamylamine (5 mg/kg) has previously been reported to induce a pseudo-abstinence syndrome in nicotine naive rats, suggesting that blockade of endogenous nAChR stimulation might produce abstinence-like signs (Malin et al 1994). Hildebrand et al (1997) also found that their saline control group showed a trend towards an increase in overall signs at a dose of 1 mg/kg mecamylamine, and in control rats in our experiment, this effect was significant compared to baseline.

The increase in overall abstinence signs observed was significantly greater in the groups receiving a subcutaneous nicotine infusion, compared to controls. Malin and colleagues observed that nicotine-infused rats administered mecamylamine displayed significantly more gasps/writhes, teeth chatter/chews, shakes/tremors and ptosis than controls (Malin et al 1994). Although we saw hardly any gasps/writhes, we did detect significant increases in chews, headshakes and ptosis in nicotine-infused rats, in agreement with this group's findings.

Hildebrand and colleagues noted that the abstinence syndrome precipitated by chlorisondamine was characterised only by a significant increase in teeth chatter and overall signs (Hildebrand et al 1997). Thus, it differed slightly from that precipitated by mecamylamine, as well as from the abstinence syndrome elicited through termination of nicotine infusion by removal of the osmotic pump. They concluded that this was likely to be due to mecamylamine acting centrally as well as peripherally, whereas chlorisondamine was only acting peripherally at the dose used in this study, although, at higher doses, chlorisondamine has also been shown to have central effects (El-Bizri & Clarke 1994a&b, Engberg & Hajos 1994).

Several research groups have modified or extended this model of nicotine withdrawal by measuring emotional/motivational changes associated with nicotine abstinence such as conditioned place aversion (Suzuki et al 1996), intracranial self-stimulation (ICSS) thresholds (Epping-Jordan et al 1998) and the startle response (Helton et al 1993). While mecamylamine produced place aversion in chronically nicotine-treated, but not in sham-operated rats (Suzuki et al 1996), it did not lead to place aversion after acute injection of nicotine (0.2 - 0.8 mg/kg). It therefore seems likely that the mecamylamine-precipitated place aversion as a result of chronic nicotine treatment reflects physical dependence on nicotine, i.e. a withdrawal sign. During nicotine withdrawal, significant increases were seen in the amplitude of the acoustic startle response for 4 days. Again, administration of 0.4 mg/kg nicotine (i.p.) prior to testing attenuated the increased reactivity observed during nicotine withdrawal (Helton et al 1993). Nicotine withdrawal induced by removal of osmotic pumps or DH β E administration in dependent rats also resulted in a significant decrease in brain

reward function for four days, as measured by elevations in brain reward thresholds (Epping-Jordan et al 1998).

Effects of vaccination on nicotine abstinence syndrome in rats

Administration of nicotine has been shown to alleviate the abstinence syndrome, decreasing the behavioural signs of withdrawal. However, in rats passively immunised with serum containing nicotine-specific antibodies, acute nicotine administration failed to reduce signs of withdrawal (Malin et al 2001). This is thought to be due to the antibodies binding nicotine and retaining it in the blood, and the authors suggested this might reduce the likelihood of relapse in abstinent smokers by rendering tobacco use less effective in relieving withdrawal. On the other hand, it might also lead to increased nicotine consumption in an attempt to overcome the effects of the anti-nicotine antibodies.

Vaccination against cotinine should reduce cotinine-mediated antagonism of nicotine's actions by binding cotinine and sequestering it in the bloodstream, thus preventing it from crossing the blood-brain-barrier. This is supported by the increases in total abstinence scores in nicotine-treated animals, which were greater in the TT-CotSH vaccinated group, than in the control vaccine group, to the extent that the difference was very nearly significant once baseline scores had been subtracted (see Figure 4.14 B). The significantly greater "chews" score in the nicotine-treated TT-CotSH, compared to the nicotine-treated TT-Cysteine group, provides further evidence that the anti-cotinine vaccine removed cotinine-mediated antagonism of nicotine (see Figure 4.14 C).

Anti-cotinine vaccination is aimed at making NRT more effective via the reduction of cotinine-mediated antagonism of nicotine's actions. This should increase the efficacy of NRT, enabling a quitter to remain abstinent from smoking tobacco whilst using less NRT (or a formulation containing a lower dose of nicotine) to alleviate the symptoms of nicotine withdrawal. As an added benefit, the exposure of the quitter to various carcinogens contained in tobacco smoke would also be reduced as a result.

Unfortunately, the anti-cotinine antibody titres achieved in this Trial were very low (~1:800; see Figure 2.10) as a result of the mislabelling of conjugates and

the resulting mis-vaccination of control animals with active vaccine and vice-versa (see section 2.3.3.3). Consequently all rats in the "control" group raised some (low) anti-CotSH titres. By giving an extra large dose (5 times the usual concentration) of the old conjugates as a final booster injection, we were able to restore the active and control groups to a certain extent (see section 2.3.3.3 & Figure 2.9); however, the titres in the active group were not very high.

We therefore picked the rats with the highest and lowest anti-CotSH antibody titres, respectively, to use for the TT-CotSH/active vaccine and TT-Cysteine/control vaccine groups in this set of experiments. This arrangement was not ideal, but it allowed us to proceed with the experiment. The question remains, whether some of the vaccination effects observed might have been greater if higher anti-cotinine antibody titres had been achieved in the active vaccine groups and the control vaccine groups had never been exposed to the immunogen.

4.6 Chapter conclusions

The in vivo experiments conducted as part of this thesis were used to study a variety of factors and behaviours involved in the acquisition and maintenance of nicotine addiction and smoking behaviour, and also to examine several aspects of nicotine withdrawal experienced during a quit attempt, as well as the effects of vaccination against cotinine on all of the above.

The ability of nicotine to act as a positive reinforcer plays a major part in the acquisition and maintenance of nicotine-taking behaviour. Despite procedural differences (cigarette smoking versus intra-venous infusion), studies of nicotine self-administration in humans and animals have reported similar responses in terms of self-administration behaviour (Rose & Corrigall 1997). Our findings are in general agreement with previous studies, and show that rats are able to acquire and maintain nicotine self-administration over a relatively long period of time (see Figures 4.7 & 4.9). Both human and animal studies of nicotine self-administration show a relatively flat bell-shaped dose-response curve for nicotine, with maximal rates or responding occurring at intermediate nicotine doses. Nicotine supplementation via non-contingent nicotine administration

suppresses nicotine self-administration behaviour in both animal models and human cigarette smokers, and administration of nicotinic antagonists also reduces responding, although human studies tended to observe a transient increase in smoking, which is thought to represent an attempt to compensate for nicotinic receptor blockade (Rose & Corrigall 1997).

The genetic background of an individual also appears to be an important factor determining the acquisition and strength of nicotine addiction, and responses to nicotine in several behavioural tasks have been shown to vary considerably between rat strains (Pescatore et al 2005). While Shoaib et al (1997) found that Sprague-Dawley (SD) and Long Evans (LE) rats (outbred strains) acquired self-administration in 2-hour daily sessions, and (inbred) Lewis (LEW) and Fisher (F344) strains failed to acquire nicotine self-administration under these conditions, Brower et al (2002) observed that LEW rats self-administered nicotine more readily than F344 or Holtzman rats, using a 23-h unlimited access setup. In the latter study, the LEW rats were also seen to be more likely to continue self-administration in the face of increasing work requirements and decreasing drug reinforcement (Brower et al 2002).

Pavlovian conditioning processes have also been implicated in the control of drug seeking and drug taking behaviours, as well as relapse following treatment for the abuse (Bevins et al 2001). Thus repeated pairing of the psychoactive effects of nicotine (unconditioned stimulus) with cues serving as conditioned stimuli is thought to result in such cues acquiring the ability to control behaviour, even in the absence of nicotine. In smokers, such cues might include throat stimulation, taste/odour of cigarettes, discrete objects associated with smoking (cigarette pack, matches/lighter, ashtray), as well as situational (contextual) cues like a bar, living room, smoking area, or vehicle (Rose & Corrigall 1997, Lazev et al 1999, Bevins et al 2001).

Conditioned locomotor sensitisation is often used to examine the behavioural processes involved in Pavlovian conditioning with nicotine, and several groups have demonstrated that the increase in locomotor activity observed with repeated nicotine administration is enhanced when drug injections are paired with a distinct context, and can even be induced by context alone (Walter & Kuschinsky 1989, Reid et al 1996, Bevins et al 2001). We were able to confirm

that repeated administration of nicotine resulted in an enhancement of the hyperlocomotion observed after acute administration, and that the effect was more pronounced in the animals that had received their nicotine injections in the testing environment (see Figures 4.1 & 4.3). However, after only four pairings of drug administration with the context, the environmental stimuli alone only induced an increase in activity compared to their unpaired controls, but locomotor activity of the nicotine-treated group was not significantly greater than that of the saline-treated animals (see Figure 4.1).

Environmental stimuli associated with availability and subjective actions of nicotine are thought to play an important role in relapse to tobacco smoking in abstinent smokers (Caggiula et al 2001, LeSage et al 2004, Liu et al 2006). Clinical studies have demonstrated that smoking-related cues produce an enhanced desire to smoke and increase the rate, intensity and time of smoking (Mucha et al 1998, Lazev et al 1999). In fact, smoking de-nicotinised cigarettes (i.e. cues alone) produced an equal amount of smoke intake and similar levels of satisfaction compared to nicotine-containing cigarettes (i.e. cues plus nicotine; Butschky et al 1995, Gross et al 1997, Rose et al 2000).

In agreement with previous studies (Shaham et al 1997, Caggiula et al 2001, LeSage et al 2004, Liu et al 2006), we were able to confirm that cues previously associated with nicotine administration are able to induce reinstatement of nicotine-seeking (lever-pressing) behaviour to a similar degree as priming doses of nicotine (Chiamulera et al 1996, Shaham et al 1997), following extinction of self-administration behaviour (see Figure 4.10).

These animal studies support clinical observations that smoking-related cues enhance the desire to smoke. Tobacco smoking (or nicotine self-administration) is particularly effective in establishing or even magnifying the incentive properties of accompanying environmental stimuli (Balfour et al 2000, Caggiula et al 2001). Indeed, environmental stimuli associated with nicotine intake appear to play a more important role in maintaining nicotine self-administration and tobacco smoking, than is the case for other drugs of abuse (Balfour et al 2000).

The ability of a drug to exert discriminable subjective effects is central to the maintenance of addiction, and plays a major part in encouraging the development of drug-seeking behaviour by directing it towards one substance rather than another. Humans are thought to abuse psychoactive drugs in order to obtain their characteristic subjective effects, and a variety of paradigms are used to study the ability of animals to identify the subjective effects of a drug. Frequently such subjective drug effects become associated with a given environment (conditioned place preference / aversion) or, in the case of conditioned taste aversion, with the taste of a flavoured solution.

Both rats and mice have been shown to display conditioned taste aversion to a flavoured solution previously paired with nicotine administration (Shoaib et al 2000, Gommans et al 2000, Shoaib et al 2002). Our findings were in agreement with these studies, and we observed that rats displayed significant conditioned taste aversion even when relatively low doses of nicotine were used during conditioning sessions (see Figures 4.12 & 4.13).

Conditioned taste aversion relies on the ability of nicotine to act as an aversive stimulus. It has been suggested that such aversive effects might be implicated in regulating drug intake and could be important in setting an upper limit to the amount of drug consumed (Shoaib et al 2003, Laviolette & van der Kooy 2004).

As well as providing positive reinforcement, nicotine has also been shown to act as a negative reinforcer by relieving the aversive withdrawal state experienced by smokers upon abstinence. This explains why the desire to relieve the discomforts of tobacco withdrawal is the most commonly cited reason for smokers to relapse during a quit attempt. So, whilst positive reinforcement appears to control acquisition and routine maintenance of the smoking habit, the abstinence syndrome and the ability of renewed smoking to provide negative reinforcement, are central to the failure of many smoking cessation attempts (Malin 2001).

A nicotine abstinence syndrome has also been characterised in rats (Malin et al 1994, Fung et al 1996, Hildebrand et al 1997), after rendering the animals nicotine-dependent by means of chronic subcutaneous infusion of nicotine. Using mecamylamine to precipitate the abstinence syndrome, we were able to demonstrate that rats pre-treated with nicotine exhibited significantly greater

abstinence scores than controls (see Figures 4.14 B & C), in agreement with the above studies.

Nicotine replacement therapy (NRT) is the most widely used medication for smoking cessation, and can roughly double the likelihood of success of a quit attempt. However, the slow continuous delivery of nicotine from NRT means that it is not as effective at relieving withdrawal symptoms as a cigarette, which can deliver a high dose of nicotine to the brain almost immediately. As a result, the majority of abstinent smokers still relapse during a cessation attempt and it is essential to improve the efficacy of NRT in order to provide better relief from the symptoms of nicotine withdrawal.

Vaccination against nicotine is being explored as a means of reducing the ability of smoking to provide negative reinforcement, by inducing the production of anti-nicotine antibodies, which prevent the drug molecules from crossing the blood-brain-barrier (LeSage et al 2006). However, the resulting lack of relief from withdrawal symptoms could lead to smokers trying to overcome the effect of the vaccine by increasing nicotine intake. A further disadvantage is that NRT would also be rendered ineffective, leading to a significant reduction in the possible treatment options available to an abstinent smoker.

Several of the anti-nicotine vaccines currently under development have been shown to affect a variety of behavioural parameters in preclinical studies in rats, similar to the ones carried out as part of this thesis (LeSage et al 2006). These studies obtained much greater antibody titres ($\geq 1:10,000$) than those achieved in our studies, at least in part through the use of the relatively aggressive Freund's adjuvant, compared to the GSK adjuvant we used (see section 1.7.3). Also, some studies employed passive immunisation, utilising a higher dose and antibody affinity than had been previously achieved in rats by vaccination; this may have further exaggerated the likely efficacy of these vaccines (LeSage et al 2006).

In contrast to the anti-nicotine vaccines, the proposed anti-cotinine vaccine is designed to make NRT more effective. The antibodies raised in response to vaccination with this vaccine should bind cotinine, the main metabolite of nicotine, retaining it in the blood stream. Preventing cotinine from crossing the

blood-brain-barrier should reduce its antagonism of nicotine's effects, increasing the ability of NRT to relieve withdrawal symptoms in abstinent smokers. It could also be used to help smokers, who are not yet ready to quit, to reduce the number of cigarettes smoked per day, thereby decreasing their exposure to the many harmful chemicals contained in cigarette smoke and allowing them to gradually wean themselves off the habit.

Initially we used the existing stock of cotinine derivative to produce conjugates; however, as the antibody titres obtained in the first three trials were comparatively low and appeared to be decreasing from one Trial to the next (see Figure 2.10), we had another batch of CotSH made. Unfortunately, the vials containing TT-CotSH (active vaccine) and TT-Cysteine (control vaccine) were mislabelled during the production of the conjugates for vaccination. As vaccinations with the old conjugates had already commenced (due to time constraints), this led to rats being immunised with the wrong conjugates (see section 2.3.3.3), and resulted in the "control group" developing (low) anti-cotinine titres, and the "active group" not developing further anti-bodies. The end result was a control group that was no longer a pure control group, and a vaccinated group that had very low titres. This meant that differences observed between control and active vaccine groups were not as large as they might have been, had greater antibody levels been achieved.

Despite the low titres achieved in the later trials, noticeable trends were observed in a number of the behavioural experiments:

- *Locomotor activity*: nicotine-induced hyperlocomotion was more pronounced and appeared sooner in the paired group vaccinated against cotinine, than in the paired group vaccinated with TT-Cysteine (see Figure 4.3). A significant 3-way interaction (pairing x treatment x vaccination) on day 3 and a main effect of vaccination on day 4, provide further evidence that the anti-cotinine vaccine might be increasing nicotine-induced locomotor activity.
- *Intravenous self-administration*: animals vaccinated against cotinine tended to have slightly higher response levels on the active lever, and therefore more infusions (see Figures 4.9 A & B), than those vaccinated

with control vaccine. In the reinstatement test, the active vaccine group showed slightly higher levels of responding on the active lever than the animals vaccinated with TT-Cysteine at the highest dose tested (see Figure 4.10 D).

- *Mecamylamine-precipitated nicotine withdrawal*: Significant main effects of vaccination were found in the post-mecamylamine total abstinence scores, both before and after subtraction of baseline scores, as well as in the “chews” category. The increase in total abstinence signs (post-mec minus baseline) in rats vaccinated against cotinine, compared to those receiving control vaccine, was very nearly significant ($p = 0.0512$), and in the “chews” category this difference reached significance.

It seems likely that, if higher anti-cotinine antibody titres could be achieved, the trends observed might well become significant effects of vaccination.

Chapter 5

**Conclusions and
future perspective**

Chapter 5

Conclusions and future perspective

5.1 Is there a need for a vaccine targeting cotinine?

Cigarette smoking is the single largest cause of preventable death and disease, and many of the 1.2 billion smokers worldwide will die prematurely as a result of their habit (Foulds et al 2004). Cessation can greatly improve health prospects, yet of the ~50% of smokers who attempt to quit each year, only a fraction (<5%) succeed, despite the use of smoking cessation aids, such as bupropion and NRT products (see Chapter 1). Current pharmacotherapy options are clearly not effective enough, as the majority of abstinent smokers still relapse. Helping smokers to quit has become a public health priority, and more efficacious cessation aids are therefore required, if this is to be achieved. Ideally, these should reduce the reinforcing properties of nicotine, but also provide some relief from withdrawal symptoms, one of the main causes of relapse in abstinent smokers.

Several anti-nicotine vaccines are being developed; these aim to break the cycle of withdrawal and reinforcement by preventing nicotine from acting at nAChRs. Nicotine-specific antibodies raised in response to vaccination bind nicotine molecules, thus stopping them from entering the brain. In abstinent smokers, this would reduce the ability of a cigarette to relieve withdrawal symptoms in the event of a relapse, thereby decreasing nicotine-mediated reinforcement of smoking. However, it would also render all NRT products ineffective since they act by delivering low levels of nicotine to alleviate the symptoms of nicotine withdrawal (see Chapter 1). This led to the proposal to target cotinine, the main metabolite of nicotine, in an immunotherapy approach to smoking cessation.

Since the start of the project, nicotine vaccines have progressed from Phase I studies in healthy non-smokers to Phase II studies in smokers. In vaccinated smokers who achieved high anti-nicotine titres, quit rates were approximately doubled; however, when those with lower titres were included in the analysis, there was no significant difference, compared to placebo (Cytos Media Release 2005). Varenicline, a partial agonist at nAChRs, was launched approximately a year ago as a new smoking cessation treatment (see Chapter 1). However, quit rates appear to be similar to those achieved with NRT, and there have recently been some concerns over the safety of varenicline in some patient groups (Freedman 2007, Kohen & Kremen 2007). There is therefore clearly still a need for a more effective treatment for nicotine dependence.

5.2 Target validation – interaction of cotinine with nAChRs

Cotinine has been reported to have weak agonist activity at nAChRs, inducing dopamine release from rat brain preparations, albeit at 1000-fold higher concentrations than nicotine (Dwoskin et al 1999, Pashmi 2004). Our results showing cotinine-induced striatal DA release are in agreement with these findings (see section 2.2). Cotinine-mediated DA release is attenuated by mecamylamine, and pre-treatment with cotinine inhibits nicotine-induced DA release from rat striatal minces (Pashmi 2004). This suggests that cotinine is acting at nAChRs, and that antagonism of nicotine's actions is likely to be due to desensitisation of nAChRs by cotinine. Removal of cotinine-mediated antagonism of nicotine's actions by targeting cotinine with specific antibodies should therefore enhance the efficacy of NRT.

Further examination of the ability of nicotinic antagonists to block cotinine-mediated DA release could help to elucidate the nAChR subtypes involved. The actions of cotinine, and interactions between nicotine and cotinine, modulating DA release could be further explored using behavioural models:

- To extend the findings in striatal minces, microdialysis techniques could be employed to characterise cotinine-induced dopamine release in vivo, as well as the effects of cotinine on DA release in response to nicotine administration. Pre-treatment with (i.v.) cotinine has previously been observed to dose-dependently

inhibit nicotine-evoked DA release in this paradigm (Sziraki et al 1999); however it remains to be determined whether cotinine administered directly into the brain via a microdialysis probe would have the same effect. The effects of nAChR antagonists on cotinine-mediated DA release also do not appear to have been evaluated.

- Possible reinforcing effects of cotinine might be examined by means of intravenous self-administration. A variety of species have been found to self-administer nicotine intravenously (Goldberg et al 1981, Risner & Goldberg 1983, Corrigan & Coen 1989, Donny et al 1995 & 1998, Shoaib et al 1997). Rats also appear to self-administer nicotine (Bardo et al 1999); however, intravenous self-administration of cotinine does not appear to have been attempted.
- The preclinical model of nicotine abstinence syndrome described in section 4.5.1 of this thesis could be used to examine the effects of cotinine on nicotine abstinence syndrome: after initiation of spontaneous abstinence (removal of osmotic pumps), the effect of cotinine administration on withdrawal could be studied. If its agonist activity at nAChRs is sufficiently strong, cotinine should alleviate the abstinence syndrome to an extent, though it is unlikely to be as effective as nicotine. However, if its primary effect is desensitisation it would have no effect or might exacerbate the syndrome. Co-administration of cotinine after initiating withdrawal could determine whether the ability of nicotine to alleviate withdrawal symptoms was reduced in the presence of cotinine.
- There have also been reports of somatic behavioural abstinence signs in mice after prolonged nicotine exposure (Isola et al 1999, Damaj 2003). Although $\beta 2^*$ nAChRs appear to be involved in the regulation of nicotine self-administration (Besson et al 2006), administration of mecamylamine in $\beta 2$ knockout mice has been shown to trigger comparable withdrawal signs as in wild-type mice, whereas mice null for the $\beta 4$ nAChR subunit display significantly milder somatic symptoms (Salas et al 2004). This raises the possibility of using knockout or other genetically engineered mouse strains to ascertain which nAChR subtypes are likely to be involved in cotinine-mediated effects.

5.3 Immune response to vaccination against cotinine

The aim of vaccination against cotinine is to raise cotinine-specific antibodies, which bind cotinine and prevent it from entering the brain and interacting with nAChRs. Achieving a high anti-cotinine antibody titre is therefore essential to determine vaccine efficacy.

The titres achieved varied with trial and some were very low (see section 2.3.3.3 & Figure 2.10), partly due to the mislabelling of the fresh conjugates used in Trials 4 & 5 (see section 2.3.2.3.4). However, even in the early trials, titres were not as high as those achieved in comparable anti-nicotine vaccine studies (Hieda et al 1997, de Villiers et al 2004, Satoskar et al 2003). Titres measured in these studies regularly exceeded 1:10,000, which is due, at least in part, to the use of the rather aggressive Freund's adjuvant to stimulate the immune response of vaccinated animals.

As explained in section 2.4, we chose to use a GSK proprietary adjuvant, developed with a view towards future use in humans, rather than Freund's adjuvant, which is too aggressive to be used in clinical studies. By using Freund's adjuvant, anti-cotinine titres in future preclinical experiments might be increased to a level sufficient to observe reliable effects of vaccination.

Preclinical studies of nicotine vaccines have used Freund's adjuvant to achieve high antibody titres; however, this may have raised unrealistic expectations about the level of immune response that might be expected in clinical studies, where Freund's adjuvant cannot be used. Initial clinical trials of anti-nicotine vaccines have shown that antibody titres vary considerably between individuals (LeSage et al 2006).

Another strategy for increasing antibody titres would be to increase the dose of conjugate (TT-CotSH) administered with each vaccination. In the studies presented in this thesis, we used a dose of 5 µg TT-CotSH per vaccination. This was based on a comparison of 3 doses (1, 5 & 25 µg) previously carried out by Pashmi (Pashmi 2004). Upon closely re-examining the data from this dose-comparison study, we found that the range of titres achieved for each dose and the low number of animals in each group (n = 5), did not justify the conclusion that the medium dose was optimal (see section 2.3.4). However, Pashmi did

observe that even the highest TT-CotSH dose, which was 5 times greater than that used in our studies, appeared to be well tolerated. It is therefore unlikely that an increase in vaccine dose administered with each injection would impact negatively on the safety profile of the anti-cotinine vaccine.

Pre-clinical studies on anti-nicotine vaccines have tended to utilise doses of 25µg of immunogen per vaccination (Hieda et al 1997, 1999 & 2000, Keyler et al 1999, Tuncok et al 2001, Satoskar et al 2003, LeSage et al 2005), with some even giving doses of up to 250 µg (Lindblom et al 2002, Carrera et al 2004), and have achieved much greater titres (see above). Increasing the dose of TT-CotSH administered with each vaccination might therefore be a relatively simple measure to increase anti-cotinine antibody titres.

In addition to studying the levels of anti-CotSH antibodies raised, we also set out to characterise the immune response to the conjugate vaccines (TT-CotSH/TT-Cysteine) further:

We determined titres of antibodies specific for the tetanus toxoid portion of both conjugates, in order to obtain a more detailed picture of the overall magnitude of the immune response. As expected, the levels of anti-TT antibodies were much greater (~10-fold) than those measured for anti-CotSH antibodies, due to the much greater immunogenicity and size of tetanus toxoid.

Pashmi had previously observed that, whilst anti-CotSH antibodies recognised cotinine, they did not interact with any of the other nicotine metabolites examined, with the exception of a small degree of cross-reactivity for norcotinine, a very minor metabolite of nicotine with no known pharmacological activity (Pashmi 2004). Using competitive ELISA techniques, we confirmed the specificity of the anti-CotSH antibodies for cotinine (see section 2.3).

Further characterisation of the immune response to anti-cotinine vaccination is likely to be advantageous. Several studies on anti-nicotine vaccines have determined the affinity of the nicotine-specific antibodies raised (Pentel et al 2000, Maurer et al 2005). As the affinity of an antibody determines the strength with which it binds its antigen, it would be desirable to measure this for the anti-cotinine antibodies, too. Knowledge of the isotype of the antibodies raised, and whether this changes over the course of the vaccination schedule, would help to

further characterise the immune response to anti-cotinine vaccination. There are several methods for determining the affinity of monoclonal antibodies or estimating the mean affinity of polyclonal antibodies; several groups studying anti-nicotine vaccines have used competitive radioimmunoassay methods to determine the affinity of the antibodies produced (Müller 1983). Unfortunately, we were unable to perform these experiments in addition to those described in this thesis, due to time constraints.

5.4 Effects of anti-cotinine vaccination on measures of nicotine dependence

In order for anti-cotinine vaccination to be a credible smoking cessation strategy, the cotinine-specific antibodies raised in response to immunisation with TT-CotSH need to have a measurable effect on parameters of nicotine dependence. We therefore performed the following studies:

In vitro: we examined whether the presence of anti-cotinine antibodies affected nicotine and cotinine distribution to blood and brain, or the upregulation of nAChRs observed following chronic nicotine exposure.

In vivo: we utilised a variety of behavioural models to explore the effects of anti-cotinine vaccination on aversive and reinforcing effects of nicotine and conditioned stimuli, and to examine whether vaccination against cotinine affects the severity of nicotine withdrawal syndrome.

5.4.1 Distribution of nicotine & cotinine

Vaccination against cotinine produces cotinine-specific antibodies (see above and Chapter 2), which circulate in the bloodstream and bind cotinine, preventing it from entering the brain and antagonising nicotine's actions. In vaccinated animals, plasma cotinine levels should therefore increase, whilst brain levels are expected to decrease, compared to controls.

We observed a significant increase in plasma cotinine concentrations in TT-CotSH vaccinated rats, compared to controls (see Figure 3.2); brain levels, however, were similar to those found in rats vaccinated with TT-Cysteine. This could have been due to contamination of brain samples with minor traces of blood during dissection. Perfusion of rats prior to taking the brain clearly

reduced such contamination; by comparison, unperfused rats had brain cotinine levels that were significantly higher than those in the control group (compare Figures 3.1 & 3.2). Nicotine distribution was not affected, providing further evidence of the specificity of the anti-cotinine antibodies.

Preclinical studies have also demonstrated the ability of anti-nicotine vaccines to affect nicotine distribution: brain nicotine concentrations were significantly reduced, whilst serum nicotine levels were significantly increased (Hieda et al 1997, Pentel et al 2000, Keyler et al 2005, Cerny et al 2002, de Villiers 2004, Satoskar et al 2003, Carrera et al 2004). The magnitude of these effects on nicotine distribution was greatest in rats with the highest antibody titres and therefore the greatest capacity to bind nicotine (Keyler et al 1999).

The modulation of nicotine distribution was also dependent on the dose of nicotine administered; with higher doses the reduction in brain nicotine was not quite as large as with smaller doses (Carrera et al 2004). When nicotine was given repeatedly or as a chronic infusion, immunisation had comparatively little effect on the accumulation of nicotine in the brain (Keyler et al 1999, Hieda et al 2000); however, the peak level of nicotine produced by each individual dose was still substantially reduced (LeSage et al 2006).

Similar effects are likely to be observed after anti-cotinine vaccination, with cotinine bound by antibodies and retained in the bloodstream until it is metabolised further (see Chapter 1) and eventually excreted. Brain cotinine levels should therefore be reduced as a result of vaccination, even during continuous exposure to nicotine, such as might occur with NRT, thereby decreasing any cotinine-mediated antagonism and improving the efficacy of NRT.

As explained above and in Chapter 2, the antibody titres achieved in our study were much lower than those measured in the nicotine vaccine studies; this would have had an impact on the ability of anti-cotinine vaccination to modulate cotinine distribution. It therefore seems likely that more significant effects would be observed, provided higher antibodies were achieved.

5.4.2 Upregulation of nAChRs

Chronic nicotine administration has been shown to result in upregulation of nAChRs in animals (Wonnacott 1990, Flores et al 1992, Rowell & Li 1997). There have also been reports of receptor upregulation in the brains of human smokers, compared to non-smokers (Benwell et al 1988, Breese et al 1997, Perry et al 1999). Our radioligand binding experiments showed an increase in [³H]epibatidine binding levels in brain homogenates from rats chronically infused with nicotine for 7 days (see Figure 3.5), but not in those given daily nicotine injections for up to 15 days (see Figures 3.3 & 3.4).

The mechanism of receptor upregulation is still unclear; however, several theories have been put forward: chronic nicotine might increase receptor assembly or decrease receptor degradation and turnover (Darsow et al 2005, Nashmi et al 2003, Wang et al 1998, Peng et al 1994). Alternatively, it might induce a conformational switch to a more easily activated high-affinity receptor (Buisson & Bertrand 2001, Vallejo et al 2005), or facilitate the assembly of subunits into pentamers (Kuryatov et al 2005, Harkness & Millar 2002). Sallette and colleagues have suggested that intracellular binding of nicotine favours the maturation of nAChRs in the endoplasmic reticulum (ER) of the secretory pathway, leading to upregulation (Sallette et al 2005). Receptor activation and signal transduction via Ca²⁺ signalling do not appear to be necessary, as the competitive antagonist DH β E also appears to upregulate nAChRs; however, binding of nicotine to nAChR subunits/receptors is likely to be required (Nashmi & Lester 2007).

Cotinine is unlikely to contribute to upregulation via nAChR binding, as it has a much lower affinity; however, it might impair nicotine-mediated upregulation by competing for binding sites. Anti-cotinine vaccination was therefore expected to further increase [³H]epibatidine binding levels, compared to controls, by reducing cotinine-mediated antagonism of nicotine; however, no effect was observed (see Figure 3.5). The titres achieved in this trial were very low due to the mislabelling of vaccine conjugates (see section 2.3.2.3.4), which could explain the absence of a vaccine effect.

This hypothesis could be examined by repeating the radioligand binding experiments in rats with sufficiently high levels of anti-cotinine antibodies. Any observed upregulation of nAChRs after nicotine administration could then be further characterised using additional radioligands to determine the effect on a variety of nAChR subtypes, possibly in combination with autoradiography techniques to explore the localisation of upregulated receptor populations. Rats could also be implanted with minipumps filled with cotinine instead of nicotine, to assess whether cotinine alone can upregulate binding sites.

5.4.3 Locomotor activity

Chronic nicotine treatment has also been shown to lead to behavioural sensitisation; locomotor activity is increased as a result of nicotine administration, and this hyperlocomotion is enhanced by repeated exposure (Clarke & Kumar 1983, Ksir et al 1987, Clarke et al 1988, Walter & Kuschinsky 1989, Vezina et al 1992, Shoaib & Stolerman 1992, Whiteaker et al 1995), which we were able to confirm (see Figure 4.3).

We also examined the effect of pairing the test environment with the actions of nicotine, and found that this association further increased nicotine-induced hyperlocomotion (see Figure 4.3), in agreement with observations previously made by several other groups (Walter & Kuschinsky 1989, Reid et al 1996, Bevins et al 2001). Environmental stimuli are thought to play an important role in smoking behaviour, and smokers often experience difficulties in maintaining abstinence due to the reinforcing effects of such stimuli, even in the absence of nicotine (Balfour et al 2000, Caggiula et al 2001, Liu et al 2006).

Passive immunisation against nicotine has been shown to block the locomotor stimulant effects of a single dose of nicotine (Pentel et al 2000), and both vaccination and passive immunisation appear to attenuate the locomotor sensitisation resulting from repeated nicotine exposure (Carerra et al 2004). We therefore studied the effects of vaccination against cotinine on locomotor activity induced by acute and repeated nicotine administration.

By removing cotinine-mediated antagonism of nicotine's actions, anti-cotinine vaccination should enhance nicotine-induced locomotor activity. We observed a trend towards increased locomotor activity over repeated nicotine exposure in TT-CotSH vaccinated rats, with nicotine-induced hyperlocomotion occurring earlier and to a greater degree than in controls (see Figure 4.3). As antibody titres in these trials were not as high as those achieved in the anti-nicotine vaccine studies mentioned above (see section 2.3.4), it is conceivable that vaccine effects that are currently trends might become significant, provided sufficiently high levels of anti-cotinine antibodies were achieved.

As well as re-examining the effects of anti-cotinine vaccination in rats with higher antibody titres, the use of microdialysis techniques in conscious freely moving rats could be considered. Such techniques would allow the simultaneous measurement of locomotor activity and in vivo DA release in immunised and control rats, which would complement the in vitro DA release measurements carried out as part of this study (see Chapter 2).

5.4.4 Intravenous self-administration

The positive reinforcement exerted by nicotine plays an important part in the development of nicotine dependence, as well as the failure of many smoking cessation attempts (Stolerman & Shoaib 1991, Rose & Corrigall 1997). Several species have been shown to self-administer nicotine (Goldberg et al 1981, Risner & Goldberg 1983, Corrigall & Coen 1989, Donny et al 1995 & 1998, Shoaib et al 1997), and the paradigm is therefore considered a relatively accurate preclinical model of nicotine dependence; it has the advantage of nicotine intake occurring as a series of small doses, controlled by the animal, rather than the experimenter, which mimics human smoking behaviour. The effects of anti-cotinine vaccination on the reinforcing properties of nicotine and conditioned stimuli associated with nicotine delivery were therefore examined using intravenous self-administration.

Anti-nicotine vaccination has been shown to reduce the percentage of rats meeting acquisition criteria, as well as decreasing response levels in rats vaccinated after acquisition of the behaviour (LeSage et al 2005). Vaccination

against nicotine also blocked the ability of nicotine to reinstate nicotine self-administration behaviour (Lindblom et al 2002).

In the experiments described in Chapter 4, rats acquired nicotine-taking behaviour (see Figure 4.7), albeit over a comparatively long period of time, and continued to self-administer the drug for several months. During this time they underwent a series of vaccinations with TT-CotSH or TT-Cysteine (see Figure 4.9). This was expected to result in increased levels of lever-pressing on the active lever in vaccinated rats, since anti-cotinine antibodies should remove cotinine-mediated inhibition of nicotine's actions. We observed some significant increases in active lever responses and infusions obtained in rats vaccinated against cotinine, despite the variability of response rates (see Figure 4.9). However, anti-cotinine vaccination did not appear to affect responding on the active lever during reinstatement induced by either cues alone, or an injection of nicotine (see Figure 4.10), although there was a tendency for immunised rats to exhibit higher levels of responding at the largest nicotine dose tested.

It is regrettable that the success of these experiments was compromised by the mislabelling of the new batch of conjugate vaccines (see section 2.3.2.3.4). This is especially unfortunate as the patency of the catheters over an extended period of time had originally been our main concern, and we found we were able to maintain patent catheters in our animals for up to 9 months in some individuals. This was most likely due to a combination of Dr Shoaib's surgical skills, the flushing of all catheters both before and after each session, and the inclusion of a small amount of heparin in the flushing solution.

Due to the low antibody titres achieved as a result of the labelling error, it is not possible to rule out a more significant effect of anti-cotinine vaccination on aspects of nicotine self-administration behaviour. The effects of vaccination against cotinine on nicotine self-administration should therefore be re-examined once immunisation parameters have been further optimised and sufficient antibody titres can be achieved. The same technique could also be used, as described in section 5.1, to determine whether cotinine is self-administered in rats.

5.4.5 Conditioned taste aversion

As well as having positive reinforcing actions, nicotine can also have aversive effects, which are thought to limit the amount self-administered by animals and smokers (Shoaib et al 2003). We used the two-bottle model of conditioned taste aversion to determine the effects of anti-cotinine vaccination on the development of taste aversion to a flavoured solution reliably paired with nicotine administration, as has been shown in non-vaccinated animals.

We were able to demonstrate the development of conditioned taste aversion (see Figures 4.12 & 4.13), even with low nicotine doses; this is in agreement with previous observations (Shoaib et al 2000 & 2002, Gommans et al 2000). However, no clear effect of vaccination was observed (see Figure 4.13); this is likely to be due to the low titres achieved in these animals as a result of the mislabelling of conjugates (see section 2.3.2.3.4).

Once high enough anti-cotinine antibody levels have been achieved, conditioned taste aversion to nicotine should therefore be re-examined, to determine whether an effect of anti-cotinine vaccination might have been missed in this trial due to the low levels of cotinine-specific antibodies. Aversive effects of nicotine might be enhanced by vaccination against cotinine, which should help to limit nicotine intake and encourage smokers to attempt to quit by gradually reducing the number of cigarettes smoked.

5.4.6 Mecamylamine-precipitated nicotine withdrawal syndrome

In dependent individuals, abstinence from nicotine precipitates a withdrawal syndrome, characterised by craving for nicotine, difficulty concentrating, depressed mood, irritability, etc. The desire to relieve the unpleasant symptoms of nicotine withdrawal is believed to be the reason high numbers of smokers relapse during a quit attempt (Malin 2001, Shiffman et al 2004).

A nicotine abstinence syndrome has also been characterised in rats (Malin et al 1992), as described in section 4.5.1. Animals were rendered nicotine dependent by chronic infusion, and behavioural somatic signs of abstinence scored following termination of nicotine exposure, either by removal of osmotic pumps or administration of nicotinic antagonists (Malin et al 1992, 1994, 1997 & 1998,

Hildebrand et al 1997, Epping-Jordan et al 1998). Abstinence scores measured were significantly higher in nicotine-treated rats, compared to controls, and our results are in agreement with these findings (see Figure 4.14).

We also examined the effect of vaccination against cotinine on the severity of the nicotine abstinence syndrome in rats. As anticipated, abstinence scores were generally higher in vaccinated animals, due to the removal of cotinine-mediated antagonism of nicotine by the cotinine-specific antibodies. In the “chews” category, the increase observed was significant (see Figure 4.14 C); furthermore, the increase in terms of total abstinence signs observed in vaccinated rats, compared to controls, was just short of significance (see Figure 4.14 B). Since anti-cotinine antibody titres achieved in this trial were very low as a result of the mislabelling of conjugate vials (see section 2.3.2.3.4), it seems likely that the effects of vaccination observed could be significant in animals with higher cotinine-specific antibodies.

Smokers experiencing withdrawal during a quit attempt often relapse because the nicotine obtained from a cigarette alleviates the withdrawal symptoms (Wise 1996, Malin 2001). NRT was developed to promote cessation by providing an alternative and less reinforcing source of nicotine; these products also do not contain the tar and carcinogens present in tobacco smoke, and are less reinforcing (see Chapter 1).

The impact of passive immunisation against nicotine on the ability of nicotine to relieve withdrawal symptoms has been studied, using the nicotine abstinence model described above. In rats injected with nicotine-specific antibodies, nicotine failed to reduce signs of withdrawal compared to controls (Malin et al 2001). Although this could reduce the likelihood of relapse by rendering tobacco use less effective in relieving withdrawal, it would also rule out the use of NRT, one of the main categories of smoking cessation products, in patients vaccinated against nicotine.

Anti-cotinine vaccination, on the other hand, is designed to increase the efficacy of NRT by removing cotinine-mediated antagonism of nicotine's actions. This hypothesis could be examined by studying the effects of vaccination against cotinine on the ability of nicotine to relieve symptoms of nicotine abstinence in rats.

The fraction of a nicotine dose converted to cotinine is much lower in rats (27%) than in humans (70%; Hieda et al 1997). Therefore, provided sufficient anti-cotinine antibody titres can be achieved, vaccination against cotinine has the potential to result in more significant effects in humans, compared to rats, making NRT much more effective and thus enabling more smokers to quit.

5.5 Possible concerns related to the clinical application of immunotherapy for nicotine dependence

A number of immunotherapy strategies targeting drugs of abuse are being developed, and some of these vaccines are already being evaluated in clinical trials (see Chapter 1). This section summarises some of the problems potential "anti-addiction" vaccines must address before they can become standard therapeutic options for the treatment of drug dependence. We discuss clinical issues specific to anti-nicotine vaccines, and explore some of the ethical dilemmas associated with "anti-addiction" vaccines generally, and those targeting nicotine dependence in particular.

5.5.1 Issues concerning anti-addiction immunotherapy generally

Conventional pharmacotherapies often act at targets such as receptors or transporter molecules, which can be widely distributed and are often involved in the control/modulation of normal physiological responses. The resulting side effects may affect the safety profile and limit the indications for which the medicine is licensed (e.g. methadone). In contrast, strategies based on vaccination are expected to have a relatively good safety profile, as the specific antibodies produced react directly with the drug of abuse, preventing drug molecules from interacting with their targets (Kosten & Owens 2005, LeSage et al 2006).

This comparative specificity of immunotherapy could also allow the approach to be used in combination with already existing pharmacotherapy options (LeSage et al 2006). The anti-cotinine vaccine, for example, might be used concurrently with NRT, and is hypothesised to improve the efficacy of NRT by removing cotinine-mediated antagonistic effects.

One of the major challenges for vaccination, as preclinical work, such as that presented in this thesis, and initial clinical trials have highlighted, is the lack of control over the titres of drug-specific antibodies achieved (Le Houezec 2005, LeSage et al 2006). In order to address this issue, booster injections are likely to be required at regular intervals throughout the treatment period. However, even if booster injections were needed every couple of months, as has been suggested (Hatsukami et al 2005, Le Houezec 2005), this dosing regimen is still minimal compared to other pharmacotherapies, where daily administration of drugs is required, which is likely to impact positively on patient compliance, leading to a greater percentage of patients completing a course of treatment.

Due to the inherent nature of the immune response, the onset of vaccine effects is likely to be slow (weeks – months), especially in comparison with other pharmacotherapy options. A course of vaccination would therefore need to be initiated several months before a quit attempt was planned, in order to ensure maximum efficacy of the treatment (Kosten & Owens 2005).

Whilst vaccination has the advantage of potentially providing long-term protection, passive immunisation results in more immediate effects. However, there are also a number of disadvantages: production of monoclonal antibodies for passive immunisation would greatly increase the expense of treatment (Kosten & Owens 2005, LeSage et al 2006). Use of polyclonal antibodies seems unlikely, as only limited quantities can be generated and there are risks of serum sickness associated with the administration of foreign proteins to humans, as well as the danger of potential transmission of animal viruses (Kosten & Owens 2005). Also, the protection provided by passive immunisation would only endure until the administered antibodies were cleared from the host system, which would necessitate more frequent dosing in order to maintain titres. On the other hand, in immune compromised individuals, such as cancer

patients, who are unable to mount an immune response of sufficient magnitude in response to vaccination, passive immunisation could provide the required antibody titres for successful immunotherapy (Kosten & Owens 2005).

Both active and passive immunisation strategies could be used in pregnant women to reduce potential harm to the unborn child and assist the mother with a quit attempt (Kosten & Owens 2005, Keyler et al 2003 & 2005). Furthermore, it is conceivable that the use of active and passive immunisation might be combined: antibodies administered by means of passive immunisation could provide initial protection whilst titres of antibodies raised as a result of vaccination were still relatively low (Kosten & Owens 2005, LeSage et al 2006).

5.5.2 Issues relating to immunotherapy strategies for the treatment of nicotine dependence

Whilst a number of anti-nicotine vaccines are being developed for the treatment of nicotine dependence, this approach could be problematic:

Vaccinated smokers might attempt to overcome the blockade of nicotine's actions mediated by the anti-nicotine antibodies through a compensatory increase in their nicotine intake, thereby reducing the ability of vaccination to break the cycle of positive reinforcement and dependence. An increase in cigarettes smoked would also expose smokers to elevated levels of tar and other carcinogens in tobacco smoke (see Chapter 1), leading to an elevated risk of cancer and other smoking-related diseases (Kosten & Owens 2005). Clinical trials have not found evidence of compensatory smoking occurring (Hatsukami et al 2005); however, the trials conducted so far have been comparatively small, and it remains to be seen whether these observations hold true in larger cohorts.

Furthermore there is a possibility that inhibition of nicotine's actions by nicotine-specific antibodies might precipitate a state of withdrawal (LeSage et al 2006), although again there is no indication from clinical trials thus far that this is the case (Hatsukami et al 2005). However, this issue could potentially pose a significant problem, as anti-nicotine vaccines do not address the feelings of craving and other withdrawal symptoms experienced as a result of nicotine abstinence.

NRT is currently one of the main treatment options for nicotine dependence (see Chapter 1). The presence of anti-nicotine antibodies in the blood of vaccinated individuals would render all forms of NRT ineffective by binding the nicotine administered therapeutically and preventing it from entering the brain (Le Houezec 2005).

Whilst neither immunotherapy strategy targets non-nicotine components of tobacco or sensory and environmental stimuli, which may contribute to nicotine dependence, vaccination against cotinine could have several advantages over anti-nicotine vaccines:

Anti-cotinine vaccination would not block the effects of nicotine and is therefore unlikely to lead to compensatory increases in the number of cigarettes smoked. In fact, it could be used in a "reduce-to-quit" approach to smoking cessation, as removing cotinine-mediated antagonism of nicotine's actions should lead to a reduction in the number of cigarettes required to achieve a desired effect. Furthermore, this would have the added bonus of reducing a smoker's exposure to other harmful tobacco constituents.

Due to its mechanism of action, the vaccination against cotinine is expected to increase the efficacy of NRT, rather than preventing the use of such a mainstay of smoking cessation pharmacotherapy. In an abstinent smoker using NRT, anti-cotinine antibodies are therefore expected to reduce the severity of any withdrawal symptoms, which may be experienced.

5.5.3 Possible ethical concerns

There are several possible scenarios in which anti-nicotine immunotherapy might be used: in current smokers to assist with a quit attempt, in ex-smokers to help maintain abstinence, and finally, in never smokers to prevent the development of nicotine dependence (Le Houezec 2005).

Use of vaccination as a therapeutic approach in the treatment of nicotine dependence is likely to be relatively uncontroversial (Hasman & Holm 2004). Patients are likely to be adults or adolescents who have been smoking for several years and are motivated to quit, but have perhaps been unable to do so in the past, or abstinent smokers experiencing difficulties in remaining abstinent.

However, there is a danger of discrimination against such individuals, by insurance companies for example (Hall 2005), since nicotine-specific antibodies resulting from vaccination would be detectable as a persistent sign of previous addiction, even if the individual had been abstinent for years. The use of passive immunisation would avoid this problem, antibodies administered would be present for a much shorter period of time (weeks rather than months) than after active immunisation (Kosten & Owens 2005); however, the cost of passive immunisation is currently prohibitively high (Kosten & Owens 2005, LeSage et al 2006).

Prophylactic immunotherapy is much more controversial. This would involve the vaccination of vulnerable individuals, who may have never taken nicotine, in order to prevent them from becoming dependent. Children and young adults are thought to be particularly susceptible to the effects of nicotine and are likely to have an increased risk of developing nicotine dependence (DiFranza et al 2002). If vaccination resulted in a lack of effect of nicotine, adolescents might be less likely to experiment in the first place, and the likelihood of becoming dependent as a result of experimentation with smoking might be reduced (Hasman & Holm 2004).

Parents might seek to have their children vaccinated against nicotine in early adolescence, before they start experimenting with tobacco (Kosten & Owens 2005, Hasman & Holm 2004, Hall 2005). However, providing adequate protection at the right point in time could prove to be extremely difficult, since the protection afforded by the current generation of anti-nicotine vaccines is not very long-lasting (without regular booster immunisations), and it is impossible to predict when an adolescent is likely to smoke their first cigarette (Kosten & Owens 2005).

Experimentation with drugs during adolescence may reflect a form of defiant behaviour, rather than pharmacological dependence, and reducing the effects of nicotine might therefore not deter the use of the drug or might promote alternative undesirable activities, such as a switch to other drugs of abuse (Kosten & Owens 2005). There is also a risk that adolescent smokers might be prompted to test vaccine efficacy by increasing nicotine intake, or that they may

turn to other drugs of abuse if the reinforcing effects of nicotine were blocked by vaccination (Kosten & Owens 2005, Hall 2005).

As minors, children would not legally be able to consent to vaccination (Hall 2005). Some have suggested that parents should be able to make such a decision on their behalf, as is already the case for a number of other childhood vaccinations (Hall 2005); however, it is difficult to compare anti-nicotine vaccines with vaccines against measles or whooping cough, for example, as nicotine dependence is not infectious (Hasman & Holm 2004). Whilst nicotine dependence might be regarded as a disease, tobacco use could be classified as a lifestyle choice, similar to consumption of alcohol or excessive quantities of fatty foods, or lack of exercise, which can also result in an increase in morbidity/mortality (Hasman & Holm 2004). On the other hand, there are known links between smoking and various diseases, such as lung cancer, emphysema, and coronary disease (Hasman & Holm 2004).

It has been argued that parental powers are limited by the child's right to an open future (Hasman & Holm 2004). Anti-nicotine vaccination is potentially irreversible and would therefore limit the future options of a child. Although smoking is often considered a noxious habit, it can also have a number of social functions in addition to its neurobehavioural effects. For some smokers the habit can provide essential pauses in a hectic daily life, whereas for others it can play an important part in social interactions. Vaccination against nicotine would not only block the neurochemical effects of the drug, but would also prevent the child from choosing to exploit the social functions of smoking (Hasman & Holm 2004).

Furthermore, there is likely to be a cost implication for prophylactic immunotherapy, as higher regulatory and safety standards would be required if anti-nicotine vaccines were to be used on healthy individuals (Hall 2005).

The anti-cotinine vaccine has the significant advantage over anti-nicotine vaccines that it is designed purely as an immunotherapy, for the treatment of nicotine dependence. It reduces cotinine-mediated antagonism of nicotine's actions by means of anti-cotinine antibodies, and could therefore be used either in a "reduce to quit" approach to smoking cessation, or in combination with NRT to enhance the efficacy of such products. Due to its mode of action it has no

utility as a vaccine to prevent the development of nicotine dependence, and the issues and problems relating to prophylactic immunotherapy therefore do not apply to this vaccine.

5.6 Conclusions

The case for an anti-cotinine vaccine as an immunotherapy is compelling; the data presented in this thesis support the hypothesis set out in Chapter 1, and there were no signs of adverse effects as a result of vaccination against cotinine. However, the efficacy of this strategy remains to be fully tested in animals generating higher titres of cotinine-specific antibodies than could be achieved here.

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